REMARKS

In the Claims:

Claims 22-26 are currently pending.

Rejections:

35 U.S.C. § 101

Claims 22-26 stand rejected under 35 U.S.C. § 101 as allegedly not being supported by either a credible, specific and substantial utility or a well established utility. In particular, the Office action alleges that "the results of the MLR assay do not support a specific and substantial utility for the claimed invention because the assay is not predictive of immune response in general, and one of ordinary skill in the art would not expect a stimulatory effect in the MLC assay to correlate to a general stimulatory effect on the immune system, absent evidence to the contrary." (Page 6 of the Office action mailed 1 November 2005). The Office action concludes that "the only immediate apparent utility for the instant invention would be further scientific characterization of PRO361 polypeptide and an antibody that binds to this protein." (Page 6 of the Office action mailed 1 November 2005).

Applicants respectfully disagree. As previously argued, Applicants maintain that the claimed antibodies, which bind the PRO 361 polypeptide, find utility in preventing suppression of an immune response. In particular, as explained at Example 34, found on page 141, Applicants disclose that the PRO361 polypeptide tested positive in the Mixed Lymphocyte Reaction (MLR) Assay, which indicates that the PRO361 polypeptide functions as an inhibitor of the proliferation of stimulated T-lymphocytes. At page 141, lines 8-9, the specification sets forth how PRO361 may be used, based on this function: "[c]ompounds which inhibit proliferation of lymphocytes are useful therapeutically where suppression of an immune response is beneficial." Thus, Applicants respectfully maintain that the asserted utility for the claimed antibodies is specific and substantial because one of ordinary skill in the art would know that antibodies to PRO361 are useful for preventing suppression of an immune response.

In rejecting Applicants' assertion of utility based on Applicants' reliance on the MLR assay, the Office applies an incorrect legal standard. When the proper legal standard is applied, it is clear that the results obtained with PRO361 in the MLR assay provide a specific and substantial utility for the claimed antibodies that bind the PRO361 polypeptide.

In interpreting the utility requirement set forth in 35 U.S.C. § 101, the CCPA, in *Nelson v. Bowler*, 626 F.2d 853, 206 USPQ (BNA) 881 (C.C.P.A. 1980), acknowledged that tests evidencing pharmacological activity of a compound may establish practical utility, *even though they may not establish a specific therapeutic use.* The court held that "since it is crucial to provide researchers with an incentive to disclose pharmaceutical activities in as many compounds as possible, we conclude adequate proof of any such activity constitutes a showing of practical utility." *Id.* at 856, 206 USPQ (BNA) at 883.

In *Cross v. Iizuka,* 753 F.2d 1047, 224 USPQ (BNA) 739 (Fed. Cir. 1985), the CAFC reaffirmed *Nelson*, and added that *in vitro* results might be sufficient to support practical utility, explaining that "*in vitro* testing, in general, is relatively less complex, less time consuming, and less expensive than *in vivo* testing. Moreover, *in vitro* results with the particular pharmacological activity are generally predictive of *in vivo* test results, *i.e.* there is a reasonable correlation there between." *Id.* at 1050, 224 USPQ (BNA) at 747. The court perceived "no insurmountable difficulty" in finding that, under appropriate circumstances, "in vitro testing, may establish a practical utility." *Id.*

The case law has also clearly established that applicants' statements of utility will be sufficient to satisfy the utility requirement of 35 U.S.C. §101, "unless there is a reason for one skilled in the art to question the objective truth of the statement of utility or its scope." *In re Langer*, 503 F.2d 1380,1391, 183 USPQ (BNA) 288, 297 (C.C.P.A. 1974). *See also In re Jolles*, 628 F.2d 1322, 206 USPQ (BNA) 885 (C.C.P.A. 1980); *In re Irons*, 340 F.2d 974, 144 USPQ (BNA) 351 (1965); *In re Sichert*, 566 F.2d 1154, 1159, 196 USPQ (BNA) 209, 212-13 (C.C.P.A. 1977). Compliance with 35 U.S.C. §101 is a question of fact. *Raytheon v. Roper*, 724 F.2d 951, 956, 220 USPQ (BNA) 592, 596 (Fed. Cir. 1983) cert. denied, 469 US 835 (1984). The evidentiary standard to be used throughout *ex parte* examination in setting forth a rejection is a preponderance of the totality of the evidence

under consideration. *In re Oetiker*, 977 F.2d 1443, 1445, 24 USPQ2d (BNA) 1443, 1444 (Fed. Cir. 1992). Thus, to overcome the presumption of truth that an assertion of utility by the applicant enjoys, the Examiner must establish that it is more likely than not that one of ordinary skill in the art would doubt the truth of the statement of utility. Only after the Examiner made a proper *prima facie* showing of lack of utility, does the burden of rebuttal shift to the applicant. The issue will then be decided on the totality of evidence.

In the present case, rejection of claims 22-26 for alleged lack of utility is improper because the Office has failed to make a *prima facie* showing of lack of utility. Indeed, although the Office action provides an explanation of the MLR assay as that assay typically is used, the reasoning for rejecting Applicants' reliance on the MLR assay provided in the Office action does not <u>alone</u> make it more likely than not that one of ordinary skill in the art would doubt the truth of Applicants' statement of utility. Making a *prima facie* showing that one of ordinary skill in the art would doubt the truth of Applicants' statement of utility is a significant burden to overcome because statistical certainty regarding Applicants' assertion of utility is not required to satisfy 35 U.S.C. § 101. *Nelson v. Bowler*, 626 F.2d at 856-857, 205 USPQ at 883-884. Indeed, where, as here, an applicant has specifically asserted that an invention has a particular utility, that assertion cannot simply be dismissed as "wrong" even where there may be some reason to question the assertion. MPEP § 2107.02.

Moreover, a 35 U.S.C. § 101 rejection should only be sustained where the asserted utility violates a scientific principle or is wholly inconsistent with contemporary knowledge in the art. *In re Gazave*, 379 F.2d 973, 978, 154 U.S.P.Q. 92, 96 (CCPA 1967). Indeed, "any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit *should be accepted as sufficient*, at least with regard to defining a 'substantial utility.'" MPEP §2107.01. As discussed below, when the legal standard is properly applied, it is clear that the Office action does not meet the burden of establishing that more likely than not one of ordinary skill in the art would question the truth of Applicants' assertion of utility.

The Office action does not allege that the asserted utility violates any scientific principle or is wholly inconsistent with contemporary knowledge but rather rejects Applicants' reliance on the MLR assay for two main reasons. First, the Office action alleges that

the MLR assay "does not appear to be predictive of general responses *in vivo*." (Page 6 of the Office action mailed November 1, 2005). Second, the Office action alleges that "[t[he specification indicates that CD4-IgG was used as a control, but it is not clear how this would control for background stimulation or provide for a measure of maximal stimulation . . . and the specification fails to provide any data or evidence of the results of the assay, therefore, one of ordinary skill in the art cannot evaluate the conclusion." (Page 6 of the Office action mailed November 1, 2005).

Neither of these bases is sufficient to establish a *prima facie* showing that one of ordinary skill in the art would doubt Applicants' assertion of utility. First, the Office action does not provide any documentary evidence establishing that MLR assay is not predictive of general immune responses *in vivo*. Indeed, the MLR is a well-established in vitro assay for assessing the ability of a test compound to stimulate or suppress T cell proliferation, and consequently the immune response of an individual. The MLR assay is described in standard textbooks, including, for example, Current Protocols in Immunology, unit 3.12; edited by Richard M. Cocio et al., National Institutes of Health, published by John Wiley & Sons, Inc., the entire contents of which are incorporated in the present specification by reference at page 141, Example 34.

As the Office action states, MLR has been extensively used and is considered to be the best *in vitro* model available to study graft-versus-host disease and graft rejection. It is well known that the transplantation of tissues or organs between individuals with MHC incompatibilities quickly activates the recipient's immune system, which then attempts to destroy the transplanted tissue or organ. Transplantation across minor histocompatibility loci generally induces a slower response. Physicians analyze the major and minor histocompatibility differences to predict the success of the graft and to adjust the aggressiveness of immunosuppressive therapy. MLR can be monitored qualitatively, for example, by following the incorporation of tritiated thymidine during DNA synthesis, by observing blast formation or by similar methods known in the art.

But in addition to being useful for determining histocompatibility, when used as in the present invention, the MLR assay is useful for detecting immunostimulatory or

immunoinhibitory activities of molecules like PRO361. Indeed, the MLR assay is widely used and is considered a standard assay for testing drug candidates that are potential immunomodulators. For example, the Gubler *et al.* reference, *PNAS,* 88:4143-4147 (1991), submitted in connection with the Fong Declaration on August 3, 2005, teaches that the MLR assay was key to identifying IL-12 as an immunostimulant of T-lymphocytes. In another example, the ability of tepoxalin, an immunomodulatory compound, to suppress graft-versus-host reaction, was first demonstrated by inhibition of proliferation of stimulated T-lymphocytes in an MLR assay. See Fung-Leung et al., *Transplantation*, 60:362-8 (1995)(submitted herewith).

In this capacity, the MLR assay is well described in the specification at page 141, Example 34, and in the Fong Declaration. In particular, at page 141, the specification describes the protocol for the MLR assay used in the present invention. For example, at lines 13-26, the specification states:

> More specifically, in one assay variant, peripheral blood mononuclear cells (PBMC) are isolated from mammalian individuals, for example a human volunteer, by leukopheresis (one donor will supply stimulator PBMCs, the other donor will supply responder PBMCs). If desired, the cells are frozen in fetal bovine serum and DMSO after isolation. Frozen cells may be thawed overnight in assay media (37° C, 5% CO_2) and then washed and resuspended to $3x10^6$ cells/ml of assay media (RPMI; 10% fetal bovine serum, 1% penicillin/streptomycin, 1% glutamine, 1% HEPES, 1% non-essential amino acids, 1% pyruvate). The stimulator PBMCs are prepared by irradiating the cells (about 3000 Rads).
>
> The assay is prepared by plating in triplicate wells a mixture of:
> 100:1 of test sample diluted to 1% or to 0.1%,
> 50:1 of irradiated stimulator cells, and
> 50:1 of responder PBMC cells.
>
> 100 microliters of cell culture media or 100 microliter of CD4-IgG is used as the control. The wells are then incubated at 37°C, 5% CO_2 for 4 days. On day 5, each well is pulsed with tritiated thymidine (1.0 mC/well; Amersham). After 6 hours the cells are washed 3 times and then the uptake of the label is evaluated.

In his declaration, Dr. Fong further explains that irradiating the PBMCs results in a population of antigen presenting cells that is mainly comprised of dendritic cells. At paragraph 7 of his declaration, Dr. Fong explains that this is an important step in the MLR assay because:

> Dendritic cells are the most potent antigen-presenting cells, which are able to "prime" naïve T cells *in vivo.* They carry on their surface high levels of major histocompatibility complex (MHC) products, the primary antigens for stimulating T-cell proliferation. Dendritic cells provide the T-cells with potent and needed accessory or costimulatory substances, in addition to giving them the T-cell maturing antigenic signal to begin proliferation and carry out their function. Once activated by dendritic cells, the T-cells are capable of interacting with other antigen presenting B cells and macrophages to produce additional immune responses from these cells.

Thus, as Dr. Fong explains at paragraph 8:

> The MLR assay of the present application is designed to measure the ability of a test substance to "drive" the dendritic cells to induce the proliferation of T-cells that are activated, or co-stimulated in the MLR, and thus identifies immune stimulants that can boost the immune system to respond to a particular antigen that may not have been immunologically active previously.

Similarly, as used in the present invention, the MLR assay is useful for measuring the ability of a test substance (or "test sample" as it is referred to in the specification at page 141) to "inhibit" the drive of dendritic cells to induce the proliferation of T-cells. Therefore, as used in the present invention, the MLR assay is neither a general predictor of immune response nor a measure of histocompatibility, but rather is a specific assay designed to test the ability of a sample, such as the polypeptide of SEQ ID NO:83 or a polypeptide encoded by the nucleic acid of SEQ ID NO:82, to inhibit the drive of dendritic cells to induce T-cell proliferation.

Second, adequate controls for using the MLR assay are both disclosed in the specification and known in the art. Further, one of ordinary skill in the art would appreciate that CD4-IgG is an antibody that might be used as a negative control in the

MLR assay. In particular, the CD4-IgG antibody can serve as a control for background stimulation by blocking or preventing activation of allogeneic responder cells.

In addition, Applicants disclose that cell culture media can be used as a control. Skilled artisans would appreciate that cell culture media would serve as a control by providing another measure of background levels. Applicants have also incorporated by reference the procedures described in Current Protocols in Immunology, unit 3.12. (*See* Coico, R (Ed.), AM et al. Proliferative Assays for T Cell Function. *Current Protocols in Immunology,* 1991 vol. 1; 3.12-1-3.12.14, submitted with the Fong Declaration August 3, 2005). Current Protocols teaches that "separate wells with control cultures should be set up that include – for each dose of responder and stimulator cells – replicate wells of responder cells with irradiated or mitomycin C-treated syngeneic stimulator cells." Current Protocols also teaches that values obtained from these controls will reflect background proliferation levels. In addition, negative controls, such as wells with either only stimulator cells or only responder cells might be included as a parameter of the MLR assay. Thus, sufficient controls for MLR are both known in the art and disclosed by Applicants such that meaningful results can be achieved using this assay.

Further, one of ordinary skill in the art would be able to evaluate the results of an MLR assay conducted using the PRO361 polypeptide because the specification makes clear that any decrease below control is a positive result, with decreases of less than or equal to 80% being preferred.

However, even if a *prima facie* case of lack of utility were made on either (or both) of the bases argued by the Examiner, the evidence submitted by Applicants clearly rebuts such a case. With regard to the Examiner's first basis for alleging lack of utility, Applicants have submitted and herein submit substantial evidence demonstrating that generally, if a test substance shows the ability to inhibit proliferation of lymphocytes in the MLR *in vitro* assay, it is more likely than not that the test substance will exhibit the same inhibitory activity *in vivo*. With regard to the Examiner's second basis for alleging lack of utility, Applicants also have demonstrated that sufficient controls were both disclosed in the specification and known in the art and that one of ordinary skill in the art

would be able to evaluate whether a particular compound tested positive in the MLR assay.

In rejecting the present invention for lack of utility, the Examiner fails to cite any references supporting the position taken in the Office action that one of ordinary skill in the art would doubt the truth of Applicants' assertion of utility based on the function of the PRO361 polypeptide demonstrated in the MLR assay. Instead, the Office action rejects Applicants' assertion of utility because allegedly, the MLR assay does not appear to be predictive of general immune responses *in vivo.* However, the claims do not require predictability of general immune responses nor is the utility of the present invention dependent on the predictability of general immune responses. Rather, the claims of the present invention are directed to anitbodies that bind to polypeptides which inhibit stimulated T-cell proliferation in the MLR assay. This characteristic of the PRO361 polypeptide is demonstrated by Example 34, which is a particular MLR assay designed to test the ability of a test sample, such as the PRO361 polypeptide, to inhibit T-cell proliferation.

Moreover, numerous *in vitro* MLR assay results which show either inhibition or stimulation of T cell proliferation by a test substance, as does the MLR in the present invention, have been confirmed *in vivo. See e.g.* the references submitted with the Amendment and Request for Reconsideration mailed August 3, 2005. In addition to the references submitted August 3, 2005, Applicants herein submit a 1993 reference by Wolos *et al.,* reporting that *in vitro* MDL 28,842 (a S-adenosyl-L-homocysteine hydrolase inhibitor, (Z)-5'-fluror-4',5'-didehydro-5'-deoxyadenosine) inhibited the generation of cytotoxic T-cells in MLR with an IC50 of less than 0.1 microM. Wolos *et al.* further report that MDL 28,842 completely inhibited the generation cytotoxic T-cells when added up to 3 days after the initiation of culture with no apparent cell toxicity. *In vivo,* MDL 28,842 inhibited the increase in popliteal lymph node weight induced by injection of allogeneic spleen cells. MDL 28,842 was also evaluated in the model of graft rejection. Skin allografts on animals given MDL 28,842 survived for 12.2 days, compared to 8.7 days for control animals. Wolos *et al.,* "Immunomodulation by an

inhibitor of S-adenosyl-L-homocysteine hydrolase: inhibition of *in vitro* and *in vivo* allogeneic responses." *Cell Immunol.* 1993 149(2):402-8 (submitted herewith).

Applicants also submit herewith a 1995 article by Fung-Leung *et al.,* reporting that tepoxalin suppressed murine T-cell proliferation in MLR and then demonstrating that oral administration of tepoxalin to mice suppressed local graft-versus host disease by about 40%. *See* Fung-Leung *et al.,* "Tepoxalin, a novel immunomodulatory compound, synergizes with CsA in suppression of graft-versus-host reaction and allogeneic skin graft rejection." *Transplantation.* 1995 60(4):362-8 (submitted herewith).

In addition, Townsend *et al.,* reported in 1998, that a CD4-CDR3 peptide analog exhibited similar results both *in vitro* in MLR assays and *in vivo* in analyses of the effectiveness of the peptide analog at preventing graft-versus-host disease. Townsend *et al.,* "Combination therapy with a CD4-CDR3 peptide analog and cyclosporine A to prevent graft-vs-host disease in a MHC-haploidentical bone marrow transplant model." *Clin Immunol. Immunopathol.* 1998 86(1):115-9 (submitted herewith). *See also* Townsend *et al.,* "Inhibitory effect of a CD4-CDR3 peptide analog on graft-versus-host disease across a major histocompatibility complex-haploidentical barrier." *Blood.* 1996 88(8):3038-47 (submitted herewith); Furukawa *et al.,* "Immunomodulation by an adenylate cyclase activator, NKH477, in vivo and vitro." *Clin Immunol. Immunopathol.* 1996 79(1):25-35 (submitted herewith).

Thus, the inhibition of T-cell proliferation by SEQ ID NO: 83 (or with the nucleic acid sequence encoding the polypeptide of SEQ ID NO: 82); as seen in the MLR results described at page 141 of the specification, would be expected to correlate with *in vivo* results. The articles discussed herein collectively teach that **in general**, <u>*in vitro* activity demonstrated in an MLR assay correlates with an *in vivo* effect</u>. Significantly, as discussed above, the law does not require the existence of a strong or linear correlation between *in vitro* and *in vivo* activity. Nor does the law require that such a correlation "always" be observed. *See e.g., Nelson v. Bowler*, 626 F.2d at 856-857, 205 USPQ at 883-884.

Hence, taken in total, as the evidence must be, in the majority of cases, the teachings in the art, as exemplified by Gubler *et al.,* Wolos *et al.*, Fung-Leung *et al.,* Townsend *et al.*, and Furukawa *et al.,* the Fong Declaration, and the references cited therewith, overwhelmingly show that an activity demonstrated in the *in vitro* MLR assay correlates with *in vivo* activity. Thus, one of skill in the art would reasonably expect in this instance, based on the MLR data demonstrating the PRO361 polypeptide has an inhibitory effect on lymphocyte proliferation *in vitro,* that PRO361 polypeptides will have an inhibitory effect on lymphocyte proliferation *in vivo.* Accordingly, Applicants have overcome any *prima facie* case of lack of utility because the claimed antibodies, which bind the PRO361 polypeptide, have utility in preventing the suppression of the immune response.

Further, as discussed above, one of ordinary skill in the art would appreciate that CD4-IgG is an antibody that might be used as a negative control by blocking or preventing activation of allogeneic responder cells. In addition, Applicants disclose that cell culture media can be used as a control. Skilled artisans would appreciate that cell culture media would serve as a control by providing a measure of background levels. Applicants have also incorporated by reference the procedures described in Current Protocols in Immunology, unit 3.12. Current Protocols teaches that "separate wells with control cultures should be set up that include – for each dose of responder and stimulator cells – replicate wells of responder cells with irradiated or mitomycin C-treated syngeneic stimulator cells." Current Protocols also teaches that values obtained from these controls will reflect background proliferation levels. In addition, negative controls, such as wells with either only stimulator cells or only responder cells might be included as a parameter of the MLR assay. Thus, sufficient controls for MLR are both known in the art and disclosed by Applicants such that meaningful results can be achieved using this assay.

In addition, the specification makes clear that any decrease below control is a positive result, with decreases of less than or equal to 80% being preferred. Further, Dr. Fong attests that it is "his considered scientific opinion that a PRO polypeptide shown to inhibit T-cell proliferation in the MLR assay where the activity is observed as 80% or

less of the control, one of skill in the art would expect to find a practical utility when an inhibition of the immune response is desired such as in autoimmune diseases." According to the Manual of Patent Examining Procedure (the "MPEP"), Office personnel are reminded that they must treat as true a statement of fact made by an applicant in relation to an asserted utility, unless countervailing evidence can be provided that shows that one of ordinary skill in the art would have a <u>legitimate basis to doubt the credibility of such a statement</u>.

Dr. Fong attests that it is "his considered scientific opinion that a PRO polypeptide shown to inhibit T-cell proliferation in the MLR assay where the activity is observed as 80% or less of the control, one of skill in the art would expect to find a practical utility when an inhibition of the immune response is desired such as in autoimmune diseases." The case law has clearly established that in considering affidavit evidence, the Examiner must consider all of the evidence of record anew. *In re Rinehart,* 531 F.2d 1084, 189 USPQ (BNA) 143 (C.C.P.A. 1976) and *In re Piasecki,* 745 F.2d. 1015, 226 USPQ (BNA) 881 (Fed. Cir. 1985). "After evidence or argument is submitted by the applicant in response, patentability is determined on the totality of the record, by a preponderance of the evidence with due consideration to persuasiveness of argument." *In re Alton,* 37 USPQ2d 1578 (Fed. Cir 1966) at 1584 quoting *In re Oetiker,* 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992)).

Furthermore, the Federal Court of Appeals held in *In re Alton*, "We are aware of no reason why opinion evidence relating to a fact issue should not be considered by an examiner." *In re Alton*, *supra.* Applicants also respectfully draw the Examiner's attention to the Utility Examination Guidelines, Part IIB, 66 Fed. Reg. 1098 (2001), which state, "Office personnel must accept an opinion from a qualified expert that is based upon relevant facts whose accuracy is not being questioned; it is improper to disregard the opinion solely because of a disagreement over the significance or meaning of the facts offered." The statement in question from an expert in the field (the Fong Declaration) states that "it is my considered scientific opinion that a PRO polypeptide shown to stimulate T-cell proliferation in the MLR assay where the activity is observed as 80% or less of the control, as specified in the present application, would be expected to find practical utility when an inhibition of

the immune response is desired, such as in autoimmune diseases." Therefore, barring evidence to the contrary regarding the above statement in the Fong Declaration, this rejection is improper under both the case law and the Utility guidelines.

Thus, the totality of the evidence demonstrates that one of ordinary skill in the art would believe that a test compound shown to inhibit T-cell proliferation in the MLR assay where the activity is observed as 80% or less of the control, would have a practical utility when an inhibition of the immune response is desired such as in autoimmune diseases. The PRO361 polypeptides tested positive in the MLR assay and therefore are useful where immunosuppression is desired. Further, antibodies to the PRO361 polypeptides are useful when prevention of immunosuppression is desired. One of ordinary skill in the art will appreciate that antibodies to PRO361 polypeptides will bind such polypeptides and thereby block or decrease the immunosuppressive activity of PRO361 polypeptides. Applicants respectfully submit that the specification provides sufficient disclosure to establish a specific, substantial, and credible utility for antibodies to the PRO361 polypeptide. Thus, Applicants respectfully request that the Examiner reconsider and withdraw this ground of rejection.

35 U.S.C. § 112, first paragraph
Enablement

The Examiner contends that because the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility, one skilled in the art would not know how to use the claimed invention.

Applicants respectfully disagree. As discussed above, the claimed antibody has the specific, substantial, and credible utility binding to a polypeptide that inhibits the proliferation of stimulated T-lymphocytes as demonstrated in the MLR assay experiment discussed in Example 34 at page 141 of the application. Applicants respectfully request the Examiner reconsider and withdraw the rejection of claims 22-26 under 35 U.S.C. § 112 ¶1 for alleged inadequate disclosure on how to use the claimed invention.

SUMMARY

Applicants believe that currently pending Claims 22-26 are patentable and respectfully request allowance thereof. The Examiner is invited to contact the undersigned attorney for Applicants via telephone if such communication would expedite prosecution of this case.

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Immunomodulation by an Inhibitor of S-Adenosyl-L-Homocysteine Hydrolase: Inhibition of *In Vitro* and *in Vivo* Allogeneic Responses

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The response of murine T cells to MHC class II determinants on allogeneic cells induces helper T cell activation and the development of cytotoxic T cells. We have recently established that an S-adenosyl-L-homocysteine hydrolase inhibitor, (Z)-5′-fluoro-4′,5′-didehydro-5′-deoxyadenosine (MDL 28,842), is a potent immunosuppressive agent which selectively inhibits T cell activation. In this report we characterize the effect of MDL 28,842 on *in vitro* and *in vivo* models of transplant rejection. *In vitro*, MDL 28,842 inhibited the generation of cytotoxic T cells in the murine mixed lymphocyte reaction with an IC_{50} of less than 0.1 μM. MDL 28,842 (1.0 μM) totally inhibited the generation of cytotoxic T cells when added up to 3 days after the initiation of culture with no apparent cell toxicity. *In vivo*, MDL 28,842 given by gavage at 5.0, 2.5, or 1.0 mg/kg/day inhibited the increase in popliteal lymph node weight induced by injection of allogeneic spleen cells into the footpad. MDL 28,842 was also evaluated in a model of graft rejection. Skin allografts on animals given MDL 28,842 at 5 mg/kg/day (ip) for the first 6 days following transplantation survived for 12.2 days, compared to 8.7 days for control animals. Cyclosporin A (CSA) given at 5.0 mg/kg/day did not prolong graft survival. The combination of MDL 28,842 and CSA was not any more effective than MDL 28,842 alone. Based on these findings, we suggest that MDL 28,842 is useful in the prevention of allograft rejection.

INTRODUCTION

The immune response of T cells to foreign MHC determinants is the basis for transplant rejection (1). Agents which inhibit T cell activation, such as cyclosporin A (CSA) and FK-506, are effective in preventing allogeneic graft (allograft) rejection (2, 3). We have recently demonstrated that a potent irreversible mechanism-based inhibitor of S-adenosyl-L-homocysteine hydrolase (AdoHcyase), (Z)-5′-fluoro-4′,5′-didehydro-5′-deoxyadenosine (MDL 28,842) (4, 5), selectively inhibits T cell activation and the production of interleukin-2 (6). Although the precise mechanism by which this compound affects T cells is unknown, MDL 28,842 increases the intracellular concentration of S-adenosyl-L-homocysteine (AdoHcy), produced during the metabolism of S-adenosylhomocysteine (AdoMet) (7). AdoHcy is a feedback inhibitor of the AdoMet-dependent transmethylation of a variety of biomolecules, among them nucleic acids

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and proteins (8–10), and therefore could affect lymphocyte activation. In humans, a congenital deficiency in another enzyme in the pathway of AdoMet metabolism, adenosine deaminase (ADA), results in severe immunodeficiency, with the most consistent feature being T cell dysfunction (11–13). MDL 28,842 inhibits T cell activation without the toxicity associated with inhibition of ADA (14, 15).

In vivo, we have recently demonstrated that MDL 28,842 is effective in two T cell-mediated experimental animal models of arthritis, collagen-induced arthritis in mice, and adjuvant arthritis in rats (16). In the present study, we demonstrate that MDL 28,842 inhibits the development of cytotoxic T cells in the mixed lymphocyte reaction, an *in vitro* correlate of transplant rejection. In addition, we demonstrate that MDL 28,842 inhibits the T cell response in the draining lymph nodes of animals immunized with allogeneic cells. In addition, MDL 28,842 is more effective than low-dose CSA in prolonging allogeneic skin graft survival in mice.

MATERIALS AND METHODS

Animals. Inbred mice, C57BL/6 (H-2^b), C3H/HeJ (H-2^k), DBA/2 (H-2^d), and BALB/C (H-2^d) were obtained from Jackson Labs (Bar Harbor, ME).

Reagents. CSA was obtained from Sandoz (Hanover, NJ). MDL 28,842 was synthesized at the Marion Merrell Dow Research Institute (4, 5).

Generation of cytotoxic T cells. Spleens were obtained from C57BL/6 and DBA/2 mice and single-cell suspensions made in Hanks' balanced salt solution (HBSS, calcium and magnesium free). Erythrocytes were lysed by treatment with Tris-buffered ammonium chloride (0.155 *M* NH_4Cl, 0.0165 *M* Tris, pH 7.2) at 37°C. The stimulator population (DBA/2) was irradiated (3000R) and both responders (3×10^7) and stimulators (3×10^7) were cocultured in T25 flasks at 37°C, 5% CO_2 in RPMI 1640 containing 10% FCS and 5×10^{-5} 2-ME (CM). MDL 28,842 was added at the beginning of culture or at times indicated. After 5 days, viable cells were recovered and assayed for cytotoxic effector cells in the ^{51}Cr release assay.

^{51}Cr release assay. P815 tumor cells (H-2^d) were used as targets. The cells were labeled with ^{51}Cr (10 $\mu Ci/10^6$ cells) at 37°C, 5% CO_2 for 1 hr in CM. Washed target cells (10^4) were added to effector cells (in triplicate) in a 96-well round-bottom plate in a final volume of 100 μl. Effector:target cell ratios began at 25:1. The plate was centrifuged at 50*g* for 5 min and incubated at 37°C, 5% CO_2 for 4 hr. Target cells were incubated with CM alone for spontaneous ^{51}Cr release and with 1% SDS for maximal release. Following incubation, the plate was centrifuged once more, the supernatant fractions were collected, and released ^{51}Cr radioactivity was determined in a Beckman gamma counter. The percentage specific lysis is expressed as:

$$100 \times \frac{\text{Experimental cpm released} - \text{spontaneous cpm released}}{\text{maximal cpm released} - \text{spontaneous cpm released}}.$$

Fluorescence microfluorometry. CD8-positive cells were stained with a fluoresceinated anti-Lyt-2 antibody (Becton-Dickinson, Mountain View, CA) and analyzed on a Coulter Epics C (Hialeah, FL) flow cytometer.

Popliteal lymph node assay. Spleen cells from BALB/C (H-2^d) and C57BL/6 mice obtained as described above were irradiated (2000R) and 10^7 cells from each strain

(in 50 μl PBS) were injected subcutaneously into each hind footpad. Seven days later animals were sacrificed and popliteal lymph nodes (LN) were removed and weighed. The net LN weight represents the weight of the LN draining the footpad injected with allogeneic cells minus the weight of the LN draining the syngeneic cell-injected footpad. MDL 28,842 at the indicated doses was given orally beginning 1 day prior to the spleen cell injections and continuing daily throughout the study.

Allogeneic skin transplantation. Skin from C57BL/6 or C3H/HeJ mice was transplanted onto BALB/C mice using a free skin grafting technique (17). Recipient animals were given compounds, intraperitoneally, 1 day before transplant, the day of transplant, and daily for either 6 days or until rejection of the graft. Mice were monitored daily for rejection. Rejection was determined by observation *in situ* and grafts were considered rejected when at least 50% of the graft's epithelium was visually destroyed. In addition, a representative graft from each group was prepared for histology and examined microscopically for rejection.

RESULTS

Effect of MDL 28,842 on the generation of cytotoxic cells in the mixed lymphocyte reaction. MDL 28,842 was added to mixed lymphocyte reaction cultures at the indicated concentrations at the initiation of culture. Five days later, viable cells were harvested and cytotoxic T cell generation was measured by the lysis of ^{51}Cr-labeled target cells. The results are presented in Fig. 1. MDL 28,842 was a potent inhibitor of cytotoxic T cell generation. The IC_{50} for this activity was less than 0.1 μM. The decreased cytotoxic T cell activity observed in cultures incubated with MDL 28,842 correlated with a decrease in the percentage of cells in the cultures expressing CD8,

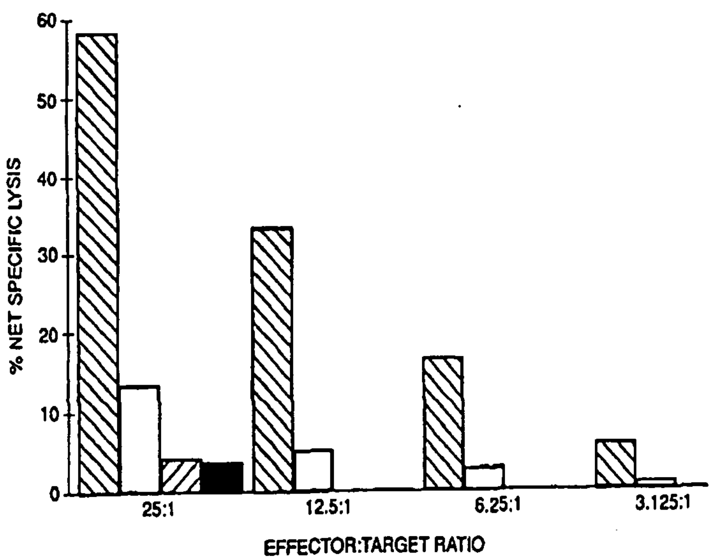

FIG. 1. Inhibition of cytotoxic T cell generation in the MLR by MDL 28,842. Cultures of C57BL/6 spleen cells (H-2^b) and irradiated DBA/2 spleen cells (H-2^d) were incubated in the absence of compound (▧), or in the presence of 0.1 (□), 1.0 (▨), or 10 μM (■) MDL 28,842. Cytotoxic T cell activity against ^{51}Cr-labeled P815 tumor cells (H-2^d) was measured 5 days later.

the cytotoxic T cell phenotype (data not shown). In subsequent experiments, MDL 28,842 was added after the initiation of culture to determine how late the compound could be added and still be effective. As shown in Table 1, the addition of MDL 28,842 (1.0 μM) up to 3 days after initiation of culture completely inhibited the generation of cytotoxic T cells. When the compound was added on Day 4 of the 5-day culture, cytotoxic T cell activity was observed, but it was substantially lower than the controls.

Inhibition of allogeneic response in the popliteal lymph nodes. BALB/C mice were dosed with MDL 28,842 by gavage at 5.0, 2.5, or 1.0 mg/kg. One day later they were injected in the footpad with 10^7 irradiated allogeneic (C57BL/6) spleen cells. The contralateral footpad received 10^7 irradiated syngeneic spleen cells. Mice were dosed with MDL 28,842 daily. Seven days after immunization, popliteal lymph nodes were isolated and weighed. In this assay, the weight correlates with the number of mononuclear cells and is related to the ongoing host versus graft T cell response (18, 19). The results are shown in Fig. 2. Treatment with all of the doses of MDL 28,842 inhibited the *in vivo* enlargement of the popliteal lymph nodes in response to allogeneic cells. Although the mean net weights observed in each treatment group suggests a dose-response, the groups were not significantly different. At the concentrations tested, all were potent inhibitors of this T-dependent response.

Effect of MDL 28,842 on skin allograft survival. Mice (8 per group) were given MDL 28,842 or CSA at 5.0 mg/kg (ip) beginning 1 day prior to skin transplantation and continuing for 6 days. The results are shown in Fig. 3. Control animals rejected skin grafts by an average of 8.7 days after transplantation. At 5 mg/kg, cyclosporin A had little effect. In contrast, MDL 28,842 prolonged skin allograft survival, with rejection delayed until 12.2 days after transplantation. Interestingly, mice given both MDL 28,842 and CSA rejected skin transplants earlier, on average, than mice given MDL 28,842 alone.

TABLE 1

Inhibition of the Development of Cytotoxic Effector Cells by MDL 28,842: Effect of Adding the Compound after Initiation of Culture

Experimental group[a]	Compound addition[b]	% Specific lysis[c] Effector:target ratio 50:1	25:1	12.5:1	6.25:1
C57BL/6	—	0	0	0	0
C57BL/6 × DBA/2	—	52.9	65.3	52.9	25.2
C57BL/6 × DBA/2	Day 0	0	0	0	0
C57BL/6 × DBA/2	Days 1, 2, and 3	0	0	0	0
C57BL/6 × DBA/2	Day 4	30.4	25.7	10.1	1.3

[a] C57BL/6 spleen cells were incubated with irradiated DBA/2 spleen cells for 5 days at which time viable cells were harvested. Recovered cells were incubated for 4 hr at 37°C with ^{51}Cr-labeled P815 tumor cells (H-2^d).

[b] MDL 28,842 (1.0 μM) was added at the indicated times after the initiation of culture.

[c] C57BL/6 spleen cells were cultured with ^{51}Cr-labeled P815 tumor cells at the indicated effector:target ratios.

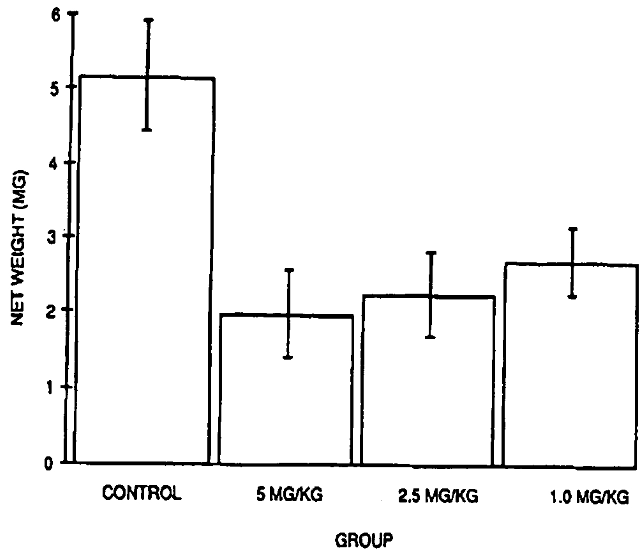

FIG. 2. MDL 28,842 inhibits lymph node enlargement stimulated by the transfer of allogeneic spleen cells. BALB/C mice (H-2^d) were injected with irradiated C57BL/6 spleen cells. Compound treatment was begun 1 day prior to injection (N = 6/group).

DISCUSSION

Inhibition of T cell activation, by agents such as cyclosporin A and, more recently, FK-506, has greatly increased the survival of tissue transplants (2, 3), although these compounds are not without side effects (20–22). MDL 28,842 is a compound which has been demonstrated to selectively inhibit mitogen-stimulated T cell activation *in vitro* and T-dependent antibody synthesis *in vivo* (6). In addition, MDL 28,842 is effective, both prophylactically and therapeutically, in the treatment of collagen-induced arthritis, a T cell-dependent experimental model of autoimmune disease (16). The precise mechanism by which MDL 28,842 mediates its effects is unclear. The compound was designed as a potent irreversible inhibitor of AdoHcyase, an enzyme in the pathway of *S*-adenosylmethionine metabolism (4, 5). Inhibition of AdoHcyase results in an accumulation of AdoHcy and the feedback inhibition of methyltransferases. These are involved in the methylation of a number of intracellular constituents, among them messenger RNA, important for cell activation (7–10). Lymphocytes seem to have a greater requirement than other cell types for methylation (23). There are other possible mechanisms by which inhibition of AdoHcyase could inhibit T cell activation. Depletion of homocysteine, the product of AdoHcyase-mediated reactions, would indirectly inhibit nucleotide synthesis by preventing the conversion of 5-methyltetrahydrofolate to tetrahydrofolate (24), which is required for folate-dependent purine and thymidylate synthesis (25). AdoHcy, which builds up in cells incubated with MDL 28,842, is also an inhibitor of PI synthase and therefore could inhibit second messenger signaling early in T cell activation (26). This possibility is not likely, since, in this report, we have shown that MDL 28,842 is effective in inhibiting the development of cytotoxic T cells *in vitro* when added as late as 3 days after the initiation of a 5-day mixed lymphocyte culture.

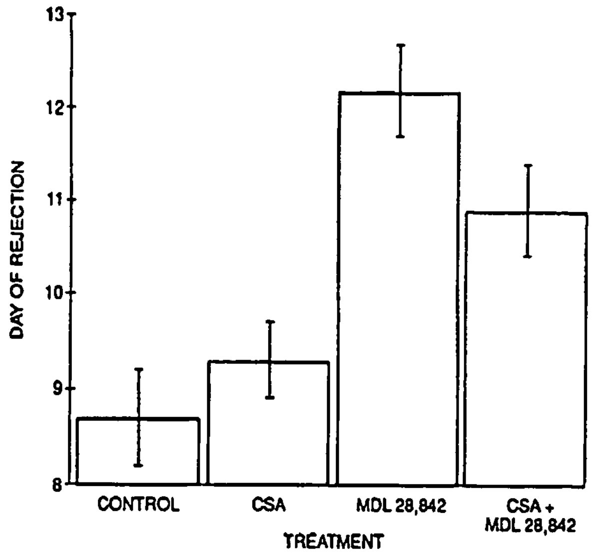

FIG. 3. MDL 28,842 prolongs allogeneic skin graft survival in mice. Comparison with CSA. The rejection of skin from C57BL/6 mice transplanted onto BALB/C mice was delayed in animals treated with MDL 28,842 at 5 mg/kg/day ip for 6 days compared to controls. CSA at 5 mg/kg/day ip did not prolong graft survival compared to controls and did not enhance the effect of MDL 28,842. (N = 8/group).

In vivo, MDL 28,842 also inhibited alloantigen-mediated T cell activation. The T cell dependent host versus graft response in the draining lymph nodes of mice injected in the footpads with irradiated allogeneic cells was reduced in animals treated with the compound. In addition, MDL 28,842 was more effective than CSA in prolonging allogeneic skin graft survival in mice. Preliminary studies suggest that MDL 28,842 also inhibits heart allograft rejection in rats (unpublished observations). The data presented previously (6, 16) and in this report demonstrate that MDL 28,842 is a potent immunosuppressive agent which inhibits T cell-mediated responses and may be useful in the prevention of transplant rejection.

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TRANSPLANTATION

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TEPOXALIN, A NOVEL IMMUNOMODULATORY COMPOUND, SYNERGIZES WITH CSA IN SUPPRESSION OF GRAFT-VERSUS-HOST REACTION AND ALLOGENEIC SKIN GRAFT REJECTION

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Tepoxalin, a dual 5-lipoxygenase and cyclooxygenase inhibitor with nonsteroidal antiinflammatory effects, has recently been shown to suppress NFκB transactivation and inhibit T cell proliferation via a mechanism very different from cyclosporine (CsA). In this report, we demonstrate that this novel immunosuppressive effect of tepoxalin is manifested in *in vivo* transplantation models. Tepoxalin suppressed murine spleen cell proliferation in a mixed lymphocyte reaction (MLR) with an IC_{50} of 1.8 μM. Coadministration of tepoxalin and CsA in MLR cultures showed an additive inhibitory effect. Oral administration of tepoxalin at 12 mg/kg/day to mice suppressed local graft-versus-host (GVH) responses by about 40% (n=10). Combination of tepoxalin and CsA at suboptimal doses synergized their immunosuppressive effects on GVH responses (n=20). In skin transplantation, the median survival time of allogeneic BALB/cByJ ($H\text{-}2^d$) mouse skin grafted onto C3H/HeJ ($H\text{-}2^k$) mice was 10.5 days (n=6), and was prolonged to 15.0 days (n=9) for recipient mice administered tepoxalin at 50 mg/kg/day. Coadministration of suboptimal doses of tepoxalin (12.5 mg/kg/day) and CsA (50 mg/kg/day) prolonged skin graft rejections dramatically (55% of the grafts survived for more than 40 days, n=9). Taken together, these results demonstrate that tepoxalin is a potent immunomodulatory compound that, when combined with CsA, provides synergistic immunosuppressive activity. The fact that tepoxalin and CsA act on different transcription factors, NFκB and NFAT respectively, might explain the synergistic suppressive effects when both compounds were used. Tepoxalin could be an important addition to the cohort of immunosuppressive therapies currently used in solid organ and bone marrow transplantations.

The immune response in transplantation, which results in graft rejection and graft-versus-host (GVH)[2] response, is primarily triggered by T cells through recognition of alloantigens (1-4). Suppression of immune response could be achieved using agents interfering with T cell activation and effector functions. The use of cyclosporine (CsA) as an immunosuppressant in transplantation has been documented (5, 6). CsA inhibits T cell activation by inhibiting the nuclear translocation of the nuclear factor NFAT (7, 8). However, CsA has associated toxicities and side effects when used at therapeutic doses (9). Compounds that suppress T cell-mediated immune response with mechanisms different from that of CsA will undoubtedly be valuable additions to the cohort of the current regiments.

Tepoxalin (5-[4-chlorophenyl]-N-hydroxy-[4-methoxyphenyl]-N-methyl-1-H-pyrazole-3-propanamide) was discovered originally as a dual inhibitor of 5-lipoxygenase (LO) and cyclooxygenase (CO) and exhibits potent nonsteroidal antiinflammatory activities in animal models of adjuvant arthritis (10-12). Recently we found that tepoxalin also inhibits OKT3-induced T cell proliferation via a mechanism very different from that of CsA (13). CsA is known to block IL-2 production after activation of T cells through TCR/CD3, whereas tepoxalin inhibits IL-2 induced signal transduction (13). An in-depth investigation of the mechanism of action reveals that tepoxalin inhibits predominantly NFκB activation (14), whereas CsA is most effective in blocking NFAT transactivation (7, 8). Because of these different mechanism of actions, a possible additive/synergistic effect of the combined tepoxalin and CsA treatment is expected. In this report, we demonstrate that tepoxalin is indeed effective in suppressing mixed lymphocyte reactions (MLR), GVH responses, and allogeneic skin graft rejections in mice. The synergistic effect of tepoxalin and CsA in immunosuppression was also studied. The possible mechanism of tepoxalin in immunosuppression and its potential clinical application are discussed.

MATERIALS AND METHODS

Mice. Inbred C57BL/6J, C3H/HeJ, and BALB/cByJ mice and $B6D2F_1/J$ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Those used in experiments were male mice at about 6-10 weeks of age that weighed 18-25 gs.

Preparation of test compounds. Tepoxalin, naproxen, and zileuton were synthesized by the R.W. Johnson Pharmaceutical Research Institute (Raritan, NJ). CsA (Sandimmune i.v.) was from Sandoz (Quebec, Canada). For MLR experiments, stock solutions of tepoxalin, naproxen, and zileuton were prepared in DMSO at 30 mM and diluted to working concentrations in culture medium at the time of experiments. DMSO at concentrations equivalent to those of the test compounds were used as controls in MLR assays. For experiments of GVH responses and skin graft rejections, micronized tepoxalin and naproxen were suspended in 0.5% methylcellulose (Sigma, St. Louis, MO) at concentrations of 5 mg/ml or lower. The vehicle control was the equivalent volume of 0.5% methylcellulose. Zileuton was dissolved in 50% polyethylene glycol 200 (Sigma, St. Louis, MO), and the corresponding vehicle control was the equivalent volume of polyethylene glycol 200. CsA was diluted in saline. All compounds were dissolved in vehicle just prior to administration to mice at volumes of

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[2] Abbreviations: CsA, cyclosporine; CO, cyclooxygenase; GVH, graft-versus-host; LO, 5-lipoxygenase; MLR, mixed lymphocyte re-

Lymphocyte proliferation assays. Single-cell suspensions from mouse spleens were washed once with PBS and then resuspended in RPMI 1640 medium supplemented with 5% fetal bovine serum and 50 μM 2-mercaptoethanol. Responder spleen cells (2.5×10^5) from C57BL/6J mice (H-2^b) were stimulated by 2.5×10^5 irradiated (2000 rads) spleen cells from B6D2F$_1$/J mice (H-$2^{b/d}$). The responder and the stimulator cells were cocultured in 250 μl medium containing various concentrations of the tested compounds in the 96-well plates (round bottom wells, Corning Inc., NY). After 5 days of stimulation, ^{3}H-thymidine was added to the cultures (0.5 μCi per well) for 4 hr. Plates were harvested using a Tomtec Harvester 96, MACH II (Tomtec Inc., Orange, CT) and samples were counted using a Wallac 1205 Betaplate scintillation counter (Pharmacia, Uppsala, Sweden).

Cell viability test. Cell viability was assessed with the MTT assay. Spleen cells from C57BL/6J mice were prepared in RPMI 1640 medium supplemented with 5% fetal bovine serum and 50 μM 2-mercaptoethanol. Spleen cells (2×10^5/well) were stimulated with immobilized anti-CD3 (Pharmingen) in the presence of tepoxalin or its vehicle DMSO in 96-well culture plates (Corning Inc., NY). The MTT assay was conducted by using the Celltiter 96 kit (Promega Corp.), which is based on the conversion of a tetrazolium salt by viable cells into a detectable blue formazan.

Graft-versus-host reactions. The GVH assay was based on the method of Dorsch and Roser (*15*). Spleen cells from C57BL/6J mice were injected subcutaneously into the footpads of B6D2F$_1$/J mice. Each footpad was injected with 8×10^6 spleen cells in 50 μl. Seven days later, the draining popliteal lymph nodes were removed, trimmed of fat and weighed. Mice injected in the footpads with saline were used as negative controls. Lymph nodes of these mice were indistinguishable from those injected with syngeneic spleen cells. Tepoxalin was administered orally and CsA was given subcutaneously to mice daily started one day before footpad injection unless otherwise specified.

Skin graft transplantation. C3H/HeJ mice (H-2^k) were anesthetized by intraperitoneal injection of 2.5% avertin (0.016 ml/g body weight). A grafting bed (about 0.3 cm×1 cm) on the mouse tail was prepared by peeling off skin carefully to avoid bleeding. Tail skin of similar size was peeled from BALB/cByJ mice (H-2^d) and then placed over the graft site in an opposite orientation according to the hair growth direction. The grafted skin was protected by a plastic tubing (diameter 0.5 cm, length 3 cm) held in place by wound clips for 5 days. Skin grafts were examined and scored daily. A graft was scored as being rejected when more than 80% of the graft was necrotic. CsA was given subcutaneously to mice daily starting one day before skin transplantation until rejection of grafts. Tepoxalin was given orally one day before transplantation and then daily starting one day after transplantation until graft rejection.

Data presentation and statistics. Data were analyzed using one-tailed Dunnett's tests. A parametric version was used if data were normally distributed as assessed by the Wilk-Shapiro test. Data which did not meet the assumptions of normality were tested using a nonparametric version of the Dunnett's test.

RESULTS

Inhibition of MLR proliferations by tepoxalin. We recently reported that tepoxalin suppresses T cell proliferation and inhibits the activity of the transcription factor NFκB (*13, 14*). NFκB activation and proliferation are critical for the initiation of an antigen specific immune response. The transcription factor NFκB is also known to be involved in regulating the expression of many target genes in an immune response (*16, 17*). The possible immunosuppressive effect of tepoxalin was therefore studied. To determine whether tepoxalin is capable of inhibiting the immune response against alloantigens, tepoxalin at various concentrations was tested in MLR proliferation assays. The assay was set up by stimulating C57BL/6J (H-2^b) mouse spleen cells with irradiated B6D2F$_1$/J (H-$2^{b/d}$) mouse spleen cells. As shown in Figure 1, tepoxalin inhibited cell proliferation in a dose-dependent fashion with an IC$_{50}$ of 1.3 μM. The inhibitory effect was not related to cell toxicity. Tepoxalin at concentrations of 25 μM or less did not affect the viability of anti-CD3 stimulated mouse spleen cells after 24 hr of treatment (Table 1). Since tepoxalin is a dual CO/LO inhibitor (*10*), the possible link of its suppression of MLR proliferation to its inhibition of CO and/or LO was studied. To address this question, the well-known CO inhibitor naproxen and the LO inhibitor zileuton were tested in parallel at doses 10-fold higher than their IC$_{50}$ for suppression of CO or LO in mice, respectively. Neither of these compounds, nor the combination of both of them, had an inhibitory effect on MLR proliferation (Fig. 1).

To further understand the mechanism of action of tepoxalin, the kinetics of tepoxalin in inhibiting MLR proliferation was compared to that of the known immunosuppressant, CsA. As shown in Table 2, the inhibitory effect was not diminished when tepoxalin was added 24–72 hr after the initiation of MLR. In contrast, CsA was effective only if it was added at the beginning of the cocultures. To determine whether tepoxalin and CsA were synergistic in inhibiting MLR proliferation, the two agents were tested in combination. Tepoxalin at 0.5 μM, 1 μM, or 2 μM was tested in combination with varying concentrations of CsA (Fig. 2). CsA alone inhibited the response in a dose-related manner with an IC$_{50}$ of 22 nM. Tepoxalin alone inhibited proliferation by 26% at 0.5 μM, by 55% at 1 μM, and by 87% at 2 μM. When tepoxalin and CsA were present at suboptimal concentrations, the inhibition was clearly additive. This additive effect was less significant at concentrations of the two drugs that were strongly inhibitory on their own.

Suppression of GVH responses by tepoxalin. The immunosuppressive effect of tepoxalin as demonstrated in MLR as-

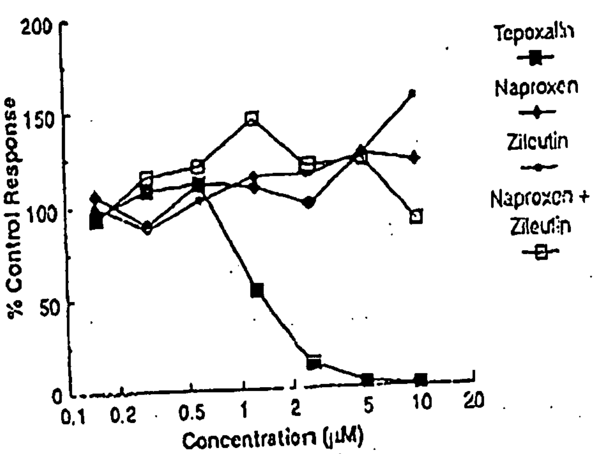

FIGURE 1. Inhibition of MLR proliferations by tepoxalin. Spleen cells from C57BL/6J mice were cocultured in triplicate wells with irradiated spleen cells from B6D2F$_1$/J mice as described in *Materials and Methods*. Varying concentrations of tepoxalin, naproxen, zileuton, or naproxen + zileuton were added to the cultures at the initiation of cultures. ^{3}H-thymidine uptake was measured on day 5. Control cultures contained DMSO diluted in a manner similar to that of the compounds. Uptake of ^{3}H-thymidine in vehicle controls was about 90,000 cpm. Percentages of control responses are calculated as percentages of (cpm of treated cultures/cpm of vehicle controls).

TABLE 1. The effective doses of tepoxalin in immunosuppression is not toxic to cells[a]

Tepoxalin (μM)	Cell viability[b]
100	53.0%
50	76.4%
25	96.5%
12.5	103.0%
6.25	115.5%
3.12	118.4%
1.56	136.5%

[a] Viability of anti-CD3 stimulated C57BL/6J spleen cells treated with tepoxalin for 24 hr was tested in the MTT viability assay.

[b] Cell viability is presented as the percentage of viable cells in tepoxalin treated sample compared with that treated with an equivalent amount of the vehicle, DMSO.

TABLE 2. Inhibitory effect of tepoxalin and CsA on MLR proliferations (% control response)[a]

	Concentration (μM)	Time of Treatment			
		0 hr	24 hr	48 hr	72 hr
Tepoxalin	1.25	64.0	52.6	67.0	30.6
	2.5	17.3	12.7	19.2	15.1
	5.0	4.7	5.2	7.8	9.0
Cyclosporine	0.021	40.6	05.0	97.1	133.6
	0.042	18.6	99.6	93.2	148.0
	0.084	5.1	61.1	84.4	119.2

[a] Different concentrations of compounds added in MLR cultures at different time points were studied. MLR assays were set up as described in *Materials and Methods*. The MLR proliferations treated with compounds were compared with their vehicle controls. ^{3}H-thymidine uptake by proliferating cells in MLR assays was measured. Percentages of control responses are calculated as percentages of (cpm of treated cultures/cpm of vehicle controls).

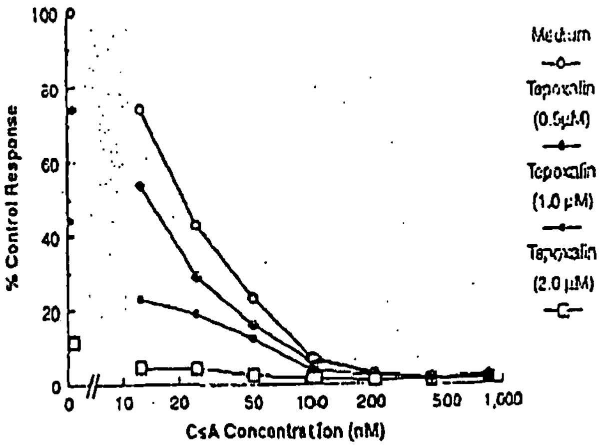

FIGURE 2. Additive inhibitory effects of tepoxalin and CsA in MLR proliferations. Proliferation of C57BL/6J mouse spleen cells after 5 days stimulation with irradiated B6D2F$_1$ spleen cells in medium containing tepoxalin at 0.5 μM, 1 μM, and 2 μM plus varying concentrations of CsA was assayed as described in *Materials and Methods*. The proliferative response in cultures containing no drugs was 80,000 cpm.

says suggests its potential use as an immunosuppressant in clinical therapy. This possible application was verified with *in vivo* murine models of transplantation. A local GVH response was performed by injecting spleen cells from the parental C57BL/6J (H-2^b) mice into the footpads of B6D2F1/J (H-2$^{b/d}$) mice. GVH responses were demonstrated by the enlargement of the draining popliteal lymph nodes in recipient mice. The lymph nodes of recipient mice increased significantly by day 2 and continued to increase in size with time. The degree of the local GVH response was measured by weighing the draining popliteal lymph nodes. The lymph nodes of tepoxalin-treated mice did enlarge on day 2 but did not change significantly later on. After 7 days of the local GVH response, lymph nodes from tepoxalin-treated mice were slightly hyperplastic, but were significantly less so than that of the untreated controls (Fig. 3A). GVH responses in mice administered tepoxalin orally at 12–50 mg/kg/day were reduced by about 40% of that in the positive control group. Consistent with the findings in mice, tepoxalin was also effective in rats, with a 30% suppression of this local GVH response at 12 mg/kg/day (data not shown). The immunosuppressive agent CsA administered subcutaneously to mice at 50 and 75 mg/kg/day was shown to suppress GVH response by 42% and 71%, respectively (Fig. 3B). The results suggest that the immunosuppressive effect of tepoxalin at 12 mg/kg/day is comparable to that of CsA at 50 mg/kg/day. To assess whether the inhibitory effect of tepoxalin on GVH responses could be obtained with other CO or LO inhibitors, naproxen and zileuton were again tested in GVH assays. No inhibition was seen with zileuton, naproxen, or a combination of the two compounds (Fig. 3C).

Since tepoxalin appears to act late in MLR assays, the effect of tepoxalin administered early and late in GVH responses was also studied. Similar to the findings in MLR proliferations, tepoxalin given to mice for a minimum of 3 days was sufficient to suppress GVH responses to an extent similar to those treated with tepoxalin throughout the 7-day course of the GVH response (Fig. 4). This short treatment with tepoxalin could be at the early (day −1 to day 1 or 4) or the late (day 4 to day 6) stage of the GVH response. The inhibitory effect of tepoxalin at the late stage of immune responses suggests its mechanism of action to be different from that of CsA. The possible synergism in immunosuppression by tepoxalin and CsA was therefore studied in GVH assays. A much stronger suppression of the GVH response was indeed found in mice treated with both tepoxalin and CsA rather than those treated with either one of the two drugs (Fig. 5). This synergistic effect was particularly significant when a low dose of tepoxalin (6 mg/kg/day) was combined with CsA.

Prolongation of skin allograft survival by tepoxalin. The time course of skin allograft rejection in mice is affected by the efficiency of the following two mechanisms: (1) the activation of T cells through recognition of specific alloantigens, and (2) the effector mechanisms mediating tissue distruction. To study the effect of tepoxalin on skin allograft survival, experimental allograft rejection was performed by grafting allogeneic BALB/cByJ (H-2^d) mouse tail-skin onto C3H/HeJ (H-2^k) recipient mice. For the first 6 days after transplantation, allografts appeared normal and their gross appearance was not different from that of syngeneic grafts. The rejection process became apparent by day 6, with signs of swelling and erythema, and quickly culminated into complete graft necrosis. Different doses of tepoxalin were tested in skin graft rejection assays. As shown in Figure 6, rejection of allografts

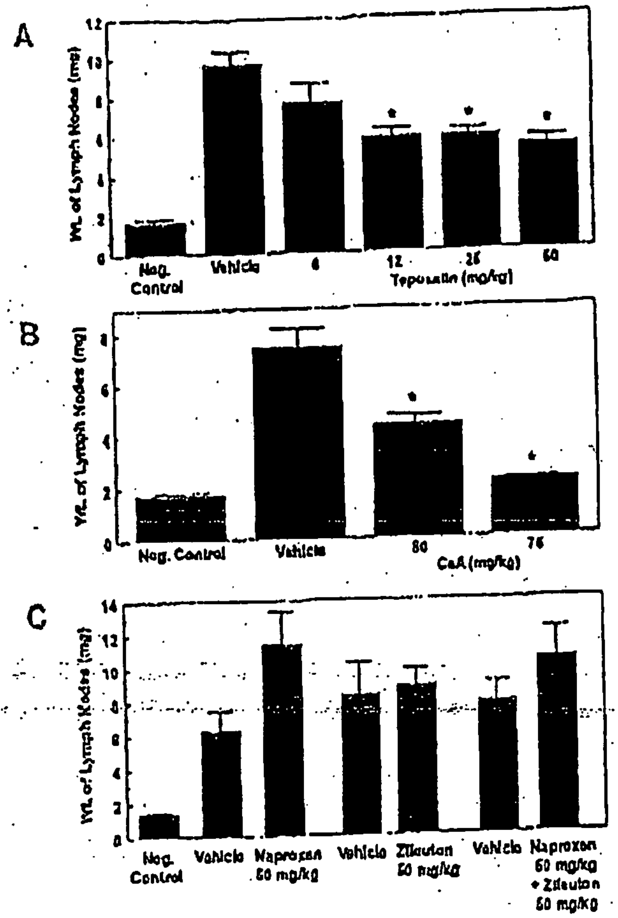

FIGURE 3. Suppression of GVH responses by tepoxalin. A local GVH response was triggered by subcutaneous injection of parental C57BL/6J spleen cells into footpads of B6D2F$_1$/J mice, and was measured by weighing the draining lymph nodes as described in *Materials and Methods*. Mice injected with saline were used as negative controls. Drugs were given to mice from day −1 to day 6 of the GVH response. (A) GVH responses in mice administered different doses of tepoxalin or vehicle control (0.5% methylcellulose) orally. Ten mice were used per group. The values from mice treated with tepoxalin at 12, 25, and 50 mg/kg/day are significantly different from the vehicle control group (Dunnett's test). Similar results were obtained from more than three repeated experiments. (B) GVH responses in mice given CsA (50 and 75 mg/kg) or vehicle control (saline) subcutaneously. Five mice were used per group. (C) GVH responses in mice given naproxen, zileuton, or the combination of the two drugs at 50 mg/kg/day orally. Mice as vehicle controls for naproxen were treated with equivalent volumes of 0.5% methylcellulose; for zileuton, they were treated with 50% polyethylene glycol 200; and for the combination of drugs, they were treated with both 0.5% methylcellulose and 50% polyethylene glycol 200. Five mice were used per group. The column bars represent the standard errors. Asterisks indicate a P value of <0.05.

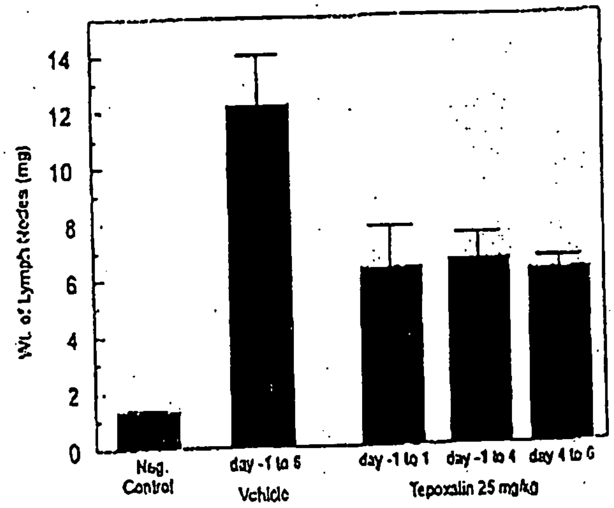

FIGURE 4. Effective suppression of mouse GVH response by short treatments with tepoxalin. The GVH response was induced by injection of C57BL/6J spleen cells into the footpads of B6D2F$_1$/J mice and was measured by weighing the draining lymph nodes as described in *Materials and Methods*. Mice injected with saline instead of spleen cells were used as negative controls. Tepoxalin (25 mg/kg) was administered orally to mice at different time schedules as shown. GVH responses in mice treated with vehicle (0.5% methylcellulose) were used as positive controls. Five mice were used per group. The column bars represent the standard errors.

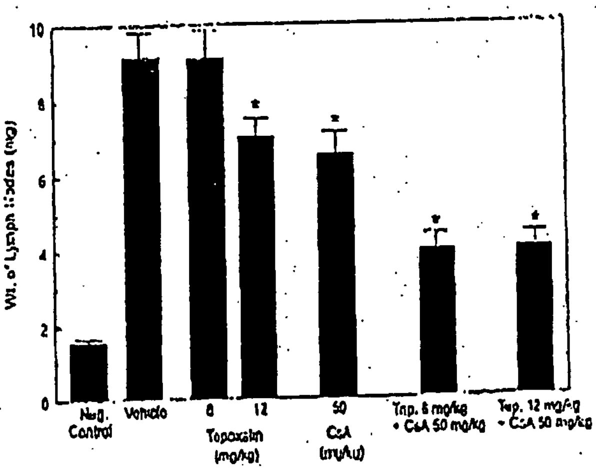

FIGURE 5. Synergistic suppression of mouse GVH responses by tepoxalin and CsA. The GVH response was induced by injection of C57BL/6J spleen cells into the footpads of B6D2F$_1$/J mice and was measured by weighing the draining lymph nodes as described in *Materials and Methods*. Mice were treated with CsA (50 mg/kg) or tepoxalin (6 or 12 mg/kg) alone, or the combination of tepoxalin (6 or 12 mg/kg) and CsA (50 mg/kg). Mice injected with spleen cells and treated with vehicles were used as positive controls. Mice injected with saline instead of spleen cells were used as negative controls. Twenty mice were used per group. The column bars represent the standard errors. Asterisks indicate a P value of <0.05. Similar results were obtained from repeated experiments.

in the placebo-treated group started on day 7. About 50% of the allografts in the placebo group were rejected on day 10. Tepoxalin at doses of 12.5 and 25 mg/kg/day did not have a significant effect in prolonging graft rejection. When tepoxalin at 50 mg/kg/day was administered to mice, a significant prolongation of skin graft rejection was observed. The median survival time of skin grafts, defined as the time point at which 50% of the grafts are rejected, was 10.6 days in the placebo-treated group and was 15.0 days for the group of mice treated with tepoxalin at 50 mg/kg/day ($P<0.05$). Furthermore, a combination of tepoxalin and CsA at low doses showed a dramatic prolongation of allogeneic skin graft rejection (Fig. 7). About 52% of the mice treated daily with

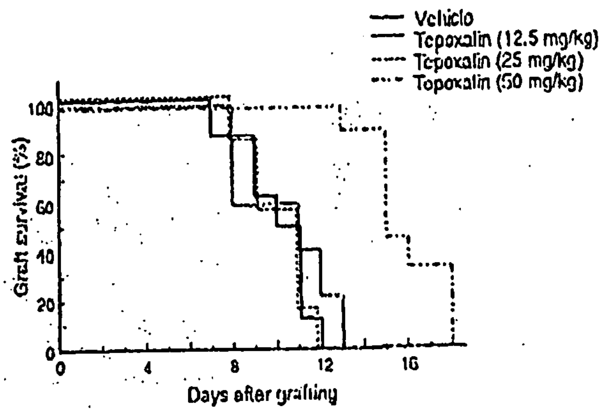

FIGURE 6. Prolongation of skin graft rejections by tepoxalin. BALB/cByJ mouse tail skin was grafted onto the tail of C3H/HeJ mice and rejection of the grafted skin was scored as described in *Materials and Methods*. Different doses of tepoxalin were administered orally to C3H/HeJ recipient mice the day before and after skin transplantation, and then daily until skin grafts were rejected. Mice given the vehicle (0.5% methylcellulose) orally were used as controls. About ten mice were used per group. Data presented were taken from one of the three repeated experiments. Results obtained from all three experiments were similar. Prolongation of skin rejection in mice treated with tepoxalin 50 mg/kg was significant ($P<0.05$, Dunnett's *t* test).

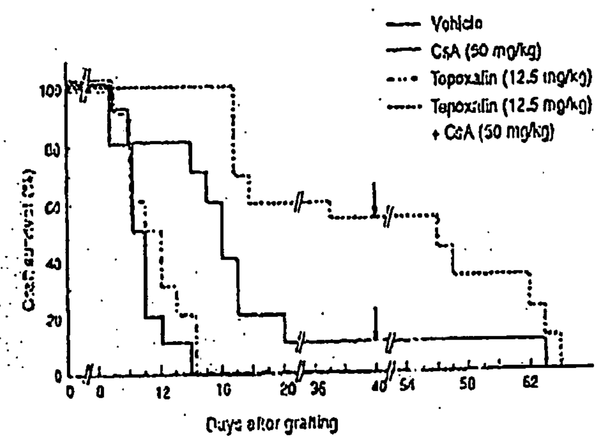

FIGURE 7. Enhanced prolongation of allogeneic skin graft rejection by tepoxalin and CsA. BALB/cByJ mouse tail skin was grafted onto the tail of C3H/HeJ mice as described in *Materials and Methods*. Tepoxalin (12.5 mg/kg) alone, CsA (50 mg/kg) alone, or tepoxalin (12.5 mg/kg) plus CsA (50 mg/kg) were administered to C3H/HeJ mice the day before and after skin transplantation, and then daily until skin grafts were rejected. Tepoxalin was given orally and CsA was given subcutaneously. For recipient mice with skin grafts surviving for more than 40 days, drug administration was discontinued from day 40, as shown by arrows. About ten mice were used per group. Enhanced prolongation of skin rejection was also observed for the combination of tepoxalin (25 mg/kg) and CsA (50 mg/kg) (data not shown).

tepoxalin (12.5 mg/kg/day) and CsA (50 mg/kg/day) retained the allogeneic skin grafts on day 40 after transplantation. To determine whether immunotolerance to skin grafts is generated by the combined drug treatment, drug dosing was discontinued after day 40 of transplantation. Skin graft rejection was noticeable on day 16 and all the grafts were rejected on day 24 after drug cessation (Fig. 7). The results suggest that the combination of tepoxalin and CsA potentiates the immunosuppressive effect, but does not induce immunotolerance to the grafts.

DISCUSSION

In this report, we demonstrate that tepoxalin is effective in suppressing the immune responses in murine models of GVH reaction and allogeneic skin graft rejection. This immunosuppressive activity is not seen with other inhibitors of CO or LO.

To study the mechanism of immunosuppression by tepoxalin, we used the *in vitro* mixed lymphocyte reaction, which measures the proliferative response of parental strain C57BL/6J spleen cells when stimulated by B6D2F$_1$/J spleen cells. Tepoxalin inhibited the alloantigen-driven proliferative response in a dose-related manner with an IC$_{50}$ of 1.3 μM and a complete inhibition at 5 μM. A similar inhibition was seen with CsA, which had an IC$_{50}$ of approximately 22 nM and a complete inhibition at about 200 nM. However, there were differences in the kinetics of the inhibitions seen with the two compounds. Tepoxalin exerted the same degree of inhibition if added any time up to 72 hr after the set-up of MLR cultures. CsA was only inhibitory if added at the initiation of the MLR cultures. IL-2 production by T cells occurs early following activation, reaching peak levels by 24 hr of culture (*18, 19*). CsA has been known for its inhibitory effect on IL-2 production (*7, 20, 21*) and is therefore expected to affect T cells during the first 24 hr of activation. The fact that tepoxalin inhibits proliferation late in MLR assays suggests its inhibition of later events in T cell activation. One possibility is that the IL-2-mediated signal transduction pathway is affected by tepoxalin, which has been shown on human lymphocytes in our previous report (*13*).

GVH disease is a common problem in bone marrow transplantation that leads to frequent morbidity and mortality (*22*). Skin grafts trigger strong immune responses and have been one of the most difficult grafts in transplantation (*3*). The immunosuppressive activity of tepoxalin was demonstrated in murine models of GVH responses and allogeneic skin graft rejections. Tepoxalin was found to inhibit GVH responses at 12 mg/kg/day and to prolong skin graft rejections at 50 mg/kg/day. The possibility that tepoxalin blocks a later event in immune response is again implicated by its suppression of GVH reaction even when it was administered to mice 4 days after the initiation of the response.

Tepoxalin is known to be a dual CO and LO inhibitor with potent antiinflammatory effects (*10*). One of the obvious questions to ask is whether its immunosuppression is due to the inhibition of the CO or LO enzymes. The involvement of CO and LO in the modulation of immune responses remains controversial. Arachidonic acid metabolites produced by these enzymes, such as prostaglandins and leukotrienes, have many biological activities, including the modulation of inflammation and immune response (*23–29*). Indeed several inhibitors of LO have been shown to prolong graft rejection in transplantation (*30–33*). However, it was noticed that those LO inhibitors with immunosuppression activity are also potent antioxidants with inhibitory effects on NFκB activity (*34, 35*). Therefore the immunoregulatory effects of these

compounds may not be directly related to inhibition of LO. We compared the effect of tepoxalin with other known LO/CO inhibitors in our studies. Naproxen (CO inhibitor) or zileuton (LO inhibitor), or their combination, did not have any effect on MLR proliferations or GVH responses. We have reported recently that tepoxalin is distinct from other CO and LO inhibitors in its inhibition of NFκB activities (*14*). NFκB is a pleiotropic transactivator of many target genes involved in immune or inflammatory responses (*16, 17*). The immunosuppressive effect of tepoxalin may be attributed to its inhibition of NFκB and not related to the general inhibition of arachidonic acid metabolism.

Taken together, these data show that tepoxalin is an effective immunosuppressive agent. Since the mechanism of tepoxalin appears to be different from CsA in immunosuppression, it suggests a possible combinational use of the two compounds in immunosuppressive therapy. Moreover, tepoxalin is devoid of ulcerogenic actions in gastrointestinal systems that are the common side effects of other NSAID drugs (*11, 12*). The LD_{50} of tepoxalin in mice and rats was more than 400 mg/kg, which is over 10-fold higher than the effective doses used in *in vivo* immunosuppression. Tepoxalin could therefore be an important addition to the existing immunosuppressive therapeutic drugs to enhance the efficacy of treatment and to reduce drug toxicity in transplantation and autoimmunity.

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TRANSPLANTATION

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COMBINED THERAPY WITH INTERLEUKIN-4 AND INTERLEUKIN-10 INHIBITS AUTOIMMUNE DIABETES RECURRENCE IN SYNGENEIC ISLET-TRANSPLANTED NONOBESE DIABETIC MICE

Analysis of Cytokine mRNA Expression in the Graft[1]

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Syngeneic pancreatic islet grafts in nonobese diabetic (NOD) mice elicit a cell-mediated autoimmune response that destroys the insulin-producing β cells in the islet graft. IL-4 and IL-10 are cytokines that inhibit cell-mediated immunity. In this study, we evaluated the effects of IL-4 and IL-10 on the survival of syngeneic pancreatic islets transplanted into diabetic NOD mice. Islet grafts survived beyond 18 days and normoglycemia was maintained in 67% (10 of 15) of mice treated with IL-4 plus IL-10, but in none (0 of 20) of vehicle-injected (control) mice. Also, 40% (6 of 15) of the mice treated with IL-4 plus IL-10 were normoglycemic at 30 days after transplantation, compared with 14% (1 of 7) of the mice treated with IL-4 alone, 8% (1 of 13) of the mice treated with IL-10 alone, and none (0 of 20) of the control mice. Histological examination of grafts at 10 days after transplantation revealed peri-islet accumulations of mononuclear leukocytes and intact islet β cells in grafts from IL-4 plus IL-10–treated mice, whereas islets were infiltrated by leukocytes and the β cell mass was greatly reduced in grafts from control mice. Polymerase chain reaction (PCR) analysis of cytokine mRNA expression in the grafts revealed higher levels of IL-2, IFNγ, and IL-10 mRNA in grafts of diabetic compared with normoglycemic control mice, whereas IFNγ and TNFα mRNA levels were significantly decreased in grafts of IL-4 plus IL-10–treated mice compared with either normoglycemic or diabetic control mice. These results suggest that T helper (Th)1 cells and their cytokine products (IL-2, IFNγ, and TNFα) may promote islet β cell destructive insulitis and autoimmune diabetes recurrence in syngeneic islet-transplanted NOD mice, and that administration of IL-4 plus IL-10 may inhibit diabetes recurrence by suppressing Th1 cytokine production in the islet grafts.

Insulin-dependent diabetes mellitus (IDDM)* results from destruction of the insulin-producing pancreatic islet β cells by the host's own immune system. Whereas it is not known what may initiate this autoimmune response against islet β cells, there is abundant evidence that IDDM is T cell–dependent (1, 2). However, it is unclear which T cells are involved and how they may lead to islet β cell destruction. A variety of immune/inflammatory cells infiltrate the pancreatic islets and constitute the insulitis lesion (3, 4). There is evidence in human patients with IDDM (5–8) and in animals with spontaneous IDDM resembling the human disease—the nonobese diabetic (NOD) mouse and the biobreeding (BB) rat (9–22)—that islet β cell destruction may involve heterogeneous effec-

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* Abbreviations: BB, Biobreeding; CFA, complete Freund's adjuvant; IDDM, insulin-dependent diabetes mellitus; NOD, nonobese diabetic; PCR, polymerase chain reaction; Th, T helper.

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Immunomodulation by an Adenylate Cyclase Activator, NKH477, *in Vivo* and *in Vitro*

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Cyclic adenosine monophosphate (cAMP) is an intracellular second messenger which modulates T cell function. NKH477 is a direct adenylate cyclase activator derived from forskolin and now under clinical investigation as a positive inotropic agent. While the immunosuppressive effects of forskolin on lymphocytes have been reported, little is known about its effects *in vivo*. In this study, we investigated whether NKH477 has immunosuppressive effects in mice, namely on cardiac allograft survival, and on the generation of cytotoxic T lymphocytes (CTL), T cell proliferation in mixed lymphocyte reaction (MLR), and production of interleukin-2 (IL-2) in MLR and in mitogen response. We assessed the effects of standard immunosuppressant cyclosporin A (CsA) on IL-2 production and on allograft survival to estimate the intensity of rejection in this acute rejection model. Saline-treated C57BL/6 (H-2^b) mice rejected DBA/2 (H-2^d) cardiac allografts with a median graft survival time of 10 days. In contrast, median graft survival was prolonged to 12 and 15 days in mice treated with NKH477 at 1 and 3 mg/kg/day, respectively ($P < 0.01$ vs control). The equivalent dose of CsA (40 mg/kg/day) to the maintenance dose after clinical cardiac transplantation prolonged median graft survival time to 15.5 days, indicating that high dose of NKH477 was as efficacious as lower dose of CsA. Addition of NKH477 to the culture medium suppressed the generation of CTL, T cell proliferation in MLR, and production of IL-2 in MLR and in mitogen response. These results suggest that NKH477 exerts a beneficial effect on murine cardiac allograft survival by modulating T cell function.

INTRODUCTION

Cyclic adenosine monophosphate (cAMP) is an intracellular second messenger which has various modulatory effects on the immune system. Forskolin, prostaglandin E_2, and other cAMP-elevating agents have been reported to have immunosuppressive effects such as decreased production of interleukin-2 (IL-2) (1, 2). An increase in $[cAMP]_i$ is also considered to have an inhibitory effect on the generation of cytotoxic T lymphocytes (CTL) (3–6), which are considered playing a role in the course of acute allograft rejection (7–10). IL-2 is involved both in the proliferation of mature T cells and in the generation of CTL (11–17).

Forskolin directly activates adenylate cyclase and has been routinely used in *in vitro* studies investigating the effect of increased $[cAMP]_i$. However, on the grounds of the low solubility of this compound in water and its poor biological availability on oral administration, it has been unclear whether increased $[cAMP]_i$ by adenylate cyclase activators has an immunosuppressive effect *in vivo*.

NKH477, (+)-(3*R*,4*aR*,5*S*,6*S*,6*aS*,10*S*,10*aR*,10*bS*)-5-acetoxy-6-(3-dimethylaminopropionyloxy)-dodeca hydro-10,10*b*-dihydroxy-3,4*a*,7,7,10a-pentamethyl-3-vinyl-1*H*-naphtho-[2,1-*b*]pyran-1-one monohydrochloride, is a new water-soluble forskolin derivative now under clinical investigation as a positive inotropic agent. Like forskolin, NKH477 directly activates the catalytic unit of adenylate cyclase and increases $[cAMP]_i$ (18, 19). It has been shown to have beneficial effects on hemodynamic states, especially on diastolic function, in an experimental model of congestive heart failure (20). The drug also has the unique characteristic of suppressing both digitalis- and adrenaline-induced ventricular arrhythmia, in contrast to phosphodiesterase inhibitors, and can be used safely with little arrhythmogenicity (21). These characteristics may be beneficial in the treatment of heart failure developing as a manifestation of acute allograft rejection.

In this study, we investigated the effects of NKH477 on cardiac allograft survival in a murine heterotopic cardiac transplantation model. We also used cyclosporin A (CsA) as a standard immunosuppressant to estimate the intensity of rejection in this acute rejection model. One-way MLR was performed to evaluate antigen-specific CTL generation and IL-2 production in consideration of the results of measurement of plasma NKH477 and M1 (equally active metabolite of NKH477) concentration. The immunosuppressive effects of NKH477 *in vitro* were compared to CsA for the inhibition of IL-2 production in MLR and in mitogen

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response, since the effects of CsA are manifested mostly through suppression of IL-2 production. We also assessed the proliferative response of splenic T cells during one-way MLR and to exogenous IL-2 by [^{3}H]thymidine incorporation.

METHODS

Mice

Male DBA/2 (H-2^d), C57BL/6 (H-2^b), and C3H/He (H-2^k) mice aged 7–9 weeks were obtained from the Shizuoka Agricultural Cooperation Association (Shizuoka, Japan), housed in stainless-steel cages with controlled 12-hr light/dark cycle and given access to standard mouse chow and water.

Reagents

NKH477 (MW 546.1) was provided as a pure powder by Nippon Kayaku Co., Ltd. (Tokyo, Japan). It was dissolved in saline and adjusted to pH 4 with 0.01 *N* HCl. Solutions were stored at 4°C. Orally available CsA (Sandimmun drink solution; Sandoz Corporation, Basel, Switzerland) was commercially obtained and diluted with olive oil. CsA for *in vitro* use was a gift from Sandoz Corp. Stock solutions of CsA were prepared at a concentration of 30 mg/kg in dimethyl sulfoxide and diluted to the required concentrations with culture medium. In *in vivo* experiments, NKH477 was given at 0.3, 1, or 3 mg/kg/day and CsA was at 5, 10, or 40 mg/kg/day, by single daily oral administration beginning on the day of transplantation. RPMI 1640, FCS, and HBSS (Hanks' balanced salt solution) were purchased from Gibco Laboratories (Grand Island, NY). Mitomycin C and concanavalin A (Con A) were obtained from Sigma Chemical Co. (St. Louis, MO). LPS from *Esherichia coli* 055:B5 was from Difco (Detroit, MI). Recombinant human IL-2 and [*methyl*-^{3}H]-thymidine were from Amersham (Buckinghamshire, UK). Anti-Thy-1.2 monoclonal antibody and low toxic rabbit serum complement for cytotoxic elimination test were from Cedarlane Laboratories (Hornby, Ontario, Canada).

Cardiac Transplantation

DBA/2 mice served as transplant donors and C57BL/6 mice as recipients. Heterotopic cardiac transplantation was performed as previously described (22, 23). In brief, donors and recipients were anesthetized with 4% chloral hydrate at 0.01 ml/g body weight ip prior to surgery. Donor hearts were perfused with heparinized saline chilled to 4°C via the inferior vena cava and harvested after ligation of the vena cavas and pulmonary veins. The donor ascending aorta and pulmonary artery were anastomosed to the recipient abdominal aorta and inferior vena cava using a microsurgical technique. Success rate was approximately 85%. Graft failures arising within the first 3 days after transplantation were excluded from experiments as technical failures. Grafts were monitored by daily abdominal palpation and regular electrocardiogram. Rejection was defined as the day of cessation of heartbeat.

Histopathological Examination

Recipients were killed 5 days after transplantation by bleeding after being anesthetized with ether. Allografts were harvested, sectioned transversely at the maximal circumference of the ventricle, and fixed in 10% buffered formalin. The graft tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Histopathological examination was conducted by two observers who were unaware of any background data. The severity of myocardial inflammation, perivascular inflammation, and necrosis was independently graded for severity by two blinded observers on a scale of 1 (low grade), 2 (moderate grade), and 3 (high grade), and scores were averaged.

Measurement of Plasma NKH477 and M1 Concentration

Seven-week-old C57BL/6 mice were orally administered NKH477 at a single daily dose 3 mg/kg for 5 days. They were anesthetized by inhalation of ether at 30 min or 1, 2, 4, 8, or 24 hr after last administration. Blood was collected by cannulation of the inferior vena cava, heparinized, and centrifuged. Collected plasma were frozen at −70°C until measurement of the concentration of NKH477 and M1 concentrations by gas chromatography/mass spectrometry. M1 is a equally active metabolite of NKH477 where one of the *N*-methyl groups of NKH477 is demethylated.

Cytotoxic T Lymphocyte (CTL) Assay (^{51}Cr Release Assay)

One-way MLR and CTL assays were performed as described previously (24, 25). Spleen cells from C57BL/6 mice (recipient strain) and DBA/2 mice (donor strain) were used as responder and stimulator cells, respectively. Responder cells at 5.6×10^6 were cocultured with 4.0×10^6 stimulator cells treated with mitomycin C (MMC) (final concentration 25 μg/ml) at 37°C for 20 min under 5% CO_2. Cells were cultured in 24-well plates in 2 ml of sensitization medium, namely complete RPMI 1640-10 (RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 5×10^{-5} *M* 2-mercaptoethanol, 10% heat-inactivated FCS) containing 1 m*M* sodium pyruvate. NKH477 (1.5×10^{-8} *M*, 4.5×10^{-8} *M*) dissolved in saline or control vehicle only (saline) was added to the experimental cultures at the initiation of culture. After one-way MLR for 5 days, CTL activity was assessed by a ^{51}Cr release assay. DBA/2 splenic target cells (donor

TABLE 1
Median Graft Survival Time

Treatment (n)	Graft survival time of all grafts (days)	Median graft survivial time (days)
Control (10)	9, 10, 10, 10, 10, 10, 10, 11, 12, 12	10
CsA 5 mg/kg/day (6)*	10, 12, 13, 13, 14, 15	13
CsA 10 mg/kg/day (6)**,†	12, 12, 13, 14, 14, 18	13.5
CsA 40 mg/kg/day (6)**,†	12, 12, 15, 16, 18, >50	15.5
NKH477 0.3 mg/kg/day (10)	9, 10, 10, 10, 11, 12, 12, 12, 14, 14	11.5
NKH477 1 mg/kg/day (10)**,†	11, 11, 11, 12, 12, 12, 14, 14, 17, 18	12
NKH477 3 mg/kg/day (10)**,†	11, 12, 13, 14, 15, 15, 16, 17, 18, 23	15

Note. Grafts survival were determined by daily abdominal palpation and regular elecrocardiogram and confirmed histologically. Rejection was defined as the day of cessation of heartbeat. Cyclosporin A (CsA) and NKH477 were orally administered at a respective dose daily.

* $P < 0.05$ vs control, ** $P < 0.01$ vs control, † $P < 0.05$ vs NKH477 0.3 mg/kg/day group. No other statistically significant differences were observed between groups.

strain) or C3H/He targets (third party) were prestimulated with 10 μg/ml of LPS for 2 days and labeled with ^{51}Cr. ^{51}Cr-labeled target cells (1.0×10^4/well) were incubated with the C57BL/6 effector cells in quadruplicate in round-bottomed 96-well tissue culture plates at an effector-to-target ratio of 100:1, 33:1, or 11:1. After incubation at 37°C for 3.5 hr in 5% CO_2 humidified air, 100 μl of supernatant was harvested from each well and radioactivity was measured using an automatic gamma scintillation counter. Spontaneous release was less than 30% of total incorporated counts. Percentage specific cytotoxicity was calculated as

$$\%\ \text{specific cytotoxicity} = \frac{(\text{cpm experiment} - \text{cpm spontaneous release})}{(\text{cpm maximal} - \text{cpm spontaneous release})} \times 100\ (\%),$$

where spontaneous and maximum cpm were obtained by culturing target cells in medium alone and in 0.5% Triton X, respectively.

Production of IL-2 in MLR and in Mitogen Response

One-way MLR was performed in 24-well plates as described above. In the first experiment, NKH477 (4.5×10^{-8} M) dissolved in saline or saline only (control) was added in a volume of 20 μl to the experimental cultures at the initiation of culture. The supernatants were harvested serially at 12, 24, 48, 72, or 120 hr after initiation and stored at −70°C until enzyme-linked immunosorbent assay (ELISA). In the second, NKH477 dissolved in saline or stock solution of CsA in dimethyl sulfoxide was diluted with RPMI 1640 and added in a

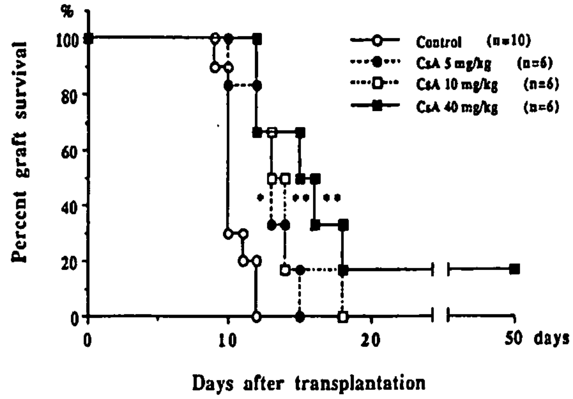

FIG. 1. Effects of cyclosporin A (CsA) on cardiac allograft survival. Oral administration of CsA at 5, 10, or 40 mg/kg/day provided slight to mild prolongation of cardiac allograft survival in comparison with control group, whereas no significant differences of efficacies were observed among treated groups. * $P < 0.05$ vs control, ** $P < 0.01$ vs control.

volume of 40 μl to the cultures at the initiation of MLR. The final concentration of NKH477 was 1.5×10^{-8}, 4.5×10^{-8}, or 13.5×10^{-8} M and CsA was 1×10^{-10}, 1×10^{-9}, 1×10^{-8}, or 1×10^{-7} M. After 48 hr MLR, the supernatants of the cultures were harvested and stored at −70°C until ELISA. In the third experiment, spleen cells (5×10^6 cells/ml) from C57BL/6 mice were incubated with 4 μg/ml of Con A in a volume of 1 ml/well for 24 hr in the presence of NKH477 or CsA at the concentrations same as the second experiment in 24-well plates. The supernatants of the cultures were harvested and stored at −70°C until ELISA. As controls, same volumes of RPMI were added to the cultures as substitutes for NKH477 and CsA. Murine IL-2 in the supernatants was quantified using ELISA systems purchased from Endogen (Boston, MA). The sensitivity of the system was >0.03 units/ml. All measurements were performed in duplicate.

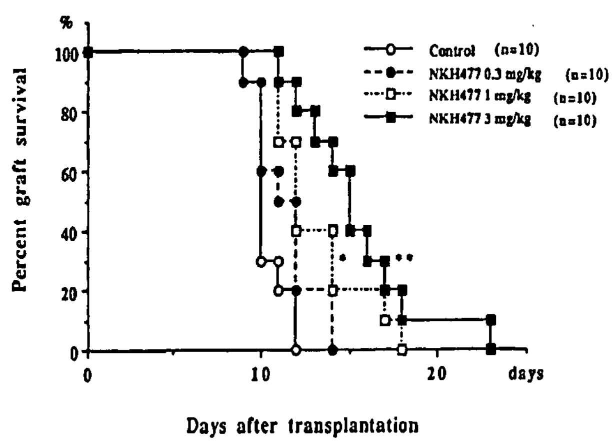

FIG. 2. Effects of NKH477 on cardiac allograft survival. Oral administration of NKH477 at 1 or 3 mg/kg/day provided slight or mild prolongation of cardiac allograft survival, whereas NKH477 at 0.3 mg/kg/day failed to prolong graft survival. * $P < 0.01$ vs control, ** $P < 0.01$ vs NKH477 0.3 mg/kg/day group.

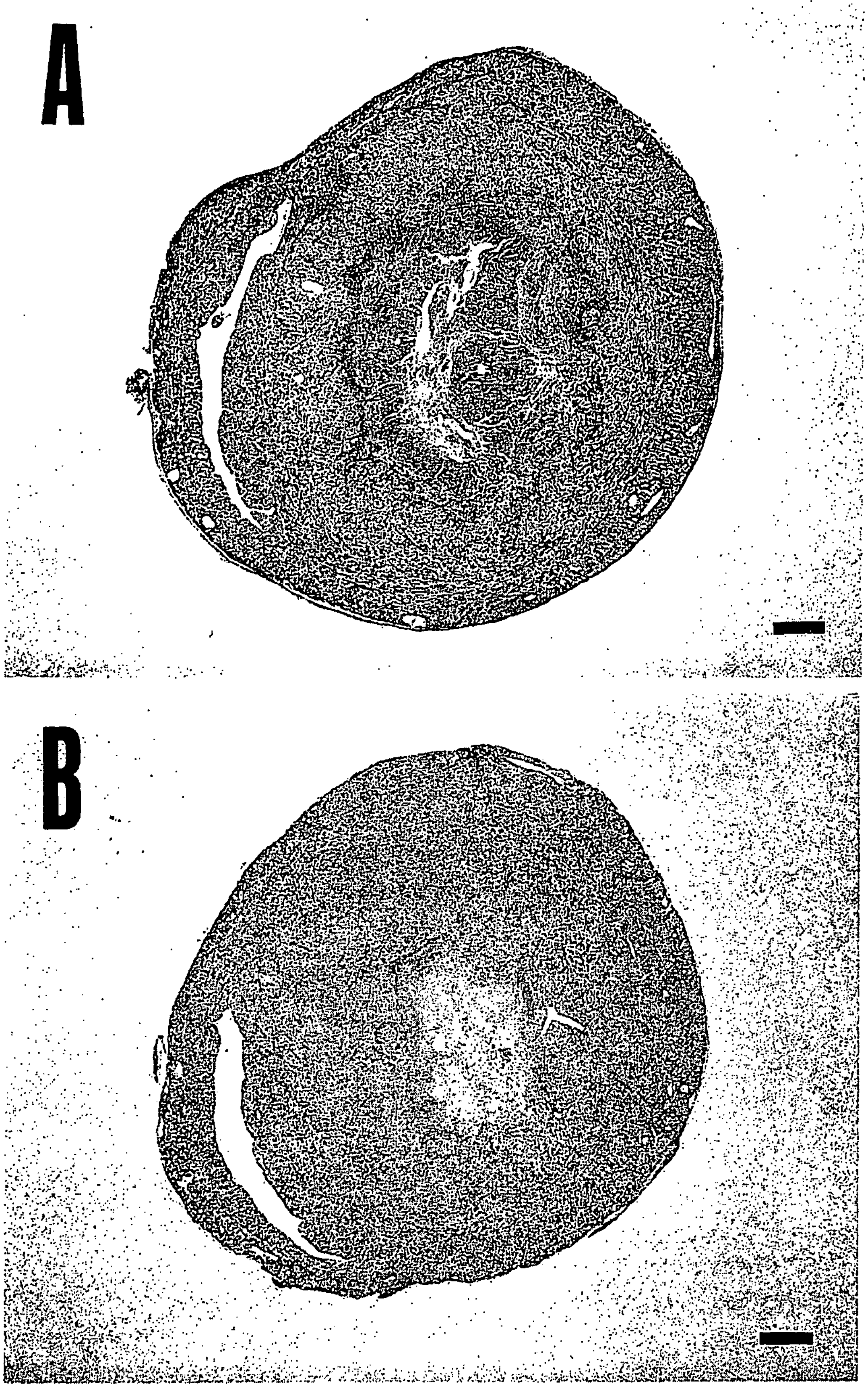

FIG. 3. Histopathology of mouse cardiac allografts. (A) Low-power photomicrograph from a vehicle-treated DBA/2 → C57BL/6 allograft. The graft was examined on Posttransplant Day 5. Note prominent interstitial mononuclear cell infiltration. (B) A DBA/2 → C57B/6 allograft treated with NKH477 at 3 mg/kg/day and examined on Posttransplant Day 5. Inflammatory changes in the graft were less severe. Bar: 500 μm. (C) High-power photomicrograph of a vehicle-treated allograft [same sample as (A), LV posterior wall]. (D) High-power photomicrograph of an NKH477-treated allograft [same sample as (B), LV posterior wall] (hematoxylin and eosin; bar: 50 μm).

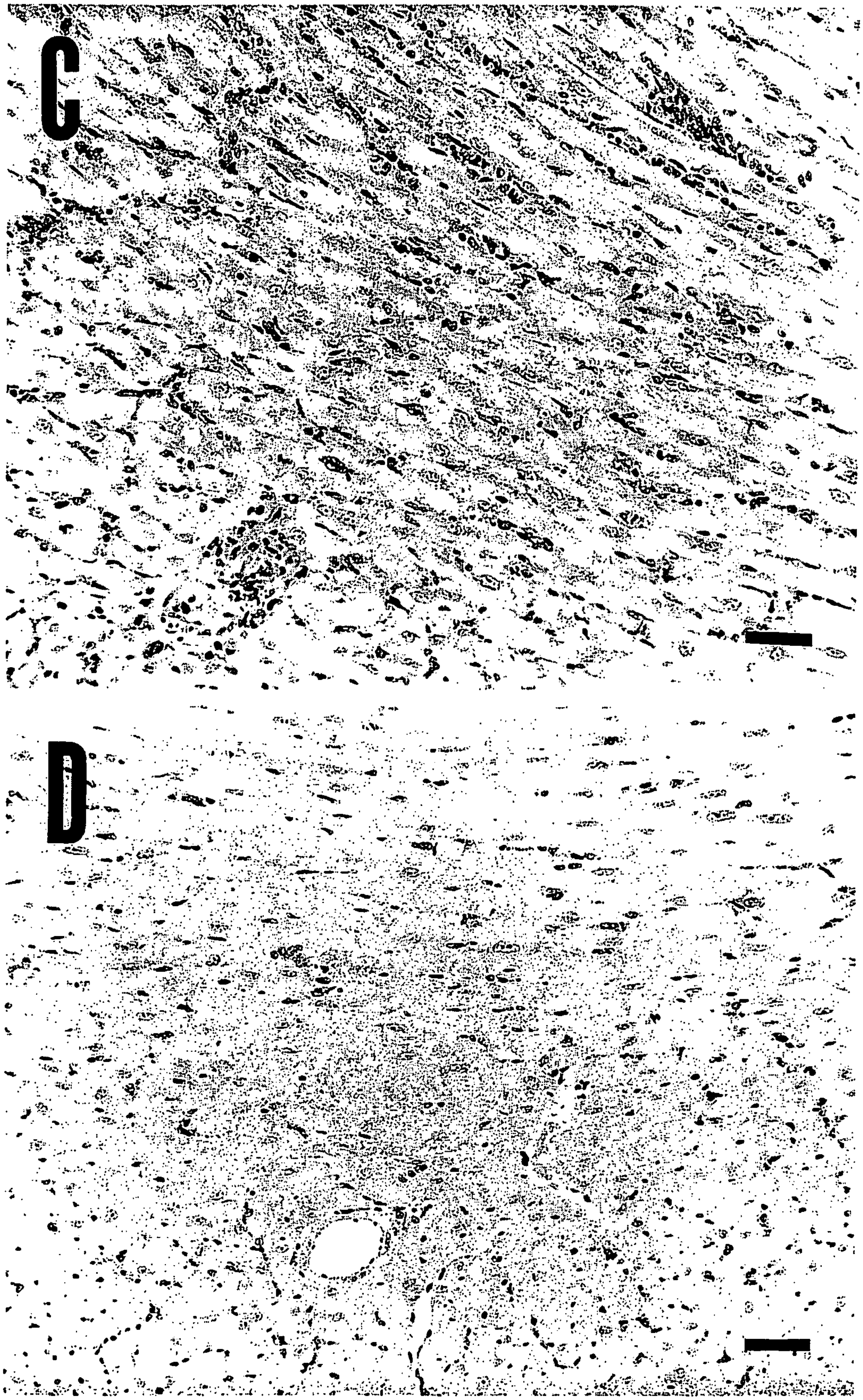

FIG. 3—*Continued*

TABLE 2
Histopathological Scores of Cardiac Allografts[a]

Treatment	n	Myocardial inflammation	Perivascular inflammation	Necrosis
Control	6	1.92 ± 0.20	1.75 ± 0.11	1.25 ± 0.11
NKH477[b]	5	1.20 ± 0.12*	1.30 ± 0.12*	1.30 ± 0.20

[a] Lesions graded as described under Methods by two blinded observers. Data are expressed as means ± SEM, and compared by Mann–Whitney U test.
[b] NKH477 was administered orally at a single daily dose of 3 mg/kg for 5 days.
* $P < 0.05$ vs control.

[^{3}H]*Thymidine Incorporation Assay*

Spleen cells from C57BL/6 mice (recipient strain) were suspended at 1.0×10^8/ml in culture medium. They were passed through nylon wool columns purchased from Wako Chemical Co. (Osaka, Japan) to enrich splenic T cells by a modification of the method established by Julius *et al.* (26) following the manufacturer's instructions. The obtained cells were subjected to cytotoxic elimination test using anti-Thy-1.2 mAb and low toxic rabbit serum complement, and shown to include 73 to 80% T cells. These enriched splenic T cells were treated in one of three ways. In the first treatment, cells were resuspended in sensitization medium and incubated at 2×10^5 cells/well with 4×10^5 cells/well MMC-treated spleen cells from DBA/2 mice in 6 wells for each treatment. NKH477 (4.5×10^{-8} *M*) diluted in RPMI 1640 or control vehicle (RPMI 1640) was added in a volume of 10 μl at the initiation of culture. On Day 5, 1 μCi of [^{3}H] thymidine was added to each well, and the cells were harvested 6 hr later. In the second treatment, cells were resuspended in complete RPMI 1640-10 and incubated at 2×10^5 cells/well in 4 wells. Recombinant human IL-2 (rhIL-2) at 1 n*M* (45 units/ml) or control vehicle (RPMI 1640), and NKH477 (4.5×10^{-8} *M*) diluted in RPMI 1640 or control vehicle (RPMI 1640) was added at the initiation of culture. After incubation for 42 hr, [^{3}H]thymidine (1 μCi) was added to each well and, after a 6-hr pulse, the cells were harvested. In the third, cells were resuspended in sensitization medium and one-way MLR was performed in 24-well culture plates as described above. On Day 4 of culture, primed cells were harvested and centrifuged. After three washes with culture medium, the viable cells were resuspended in cRPMI 1640-10 and incubated at 2×10^5/well in 5 wells with or without 1 n*M* of rhIL-2 for 18 hr. Then, after a 6-hr pulse with [^{3}H]thymidine (1 μCi/well), the cells were harvested. For all harvested cells, [^{3}H]thymidine incorporation was assessed by scintillation counting. All cultures except those for priming MLR were performed in a volume of 200 μl in round-bottomed 96-well plates.

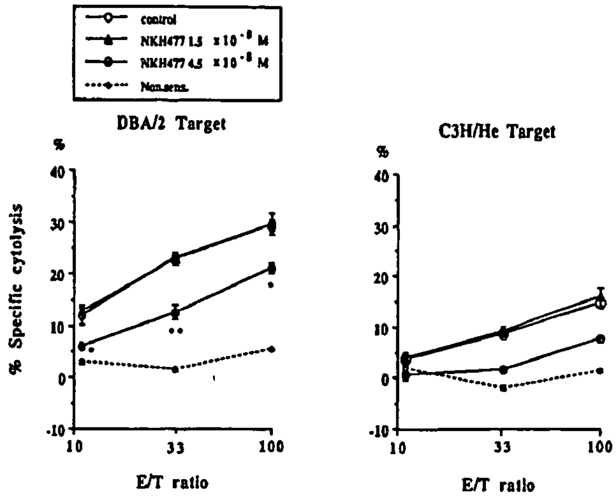

FIG. 5. Inhibition of CTL generation *in vitro* by treatment with NKH477. Splenocytes from C57BL/6 mice were stimulated with DBA/2 cells for 5 days, and then tested against DBA/2 or C3H/He cells. The graphs represent cytolytic activities at the effector to target ratios of 11, 33, and 100. NKH477 was added to the medium during the 5-day coculture at a concentration of 1.5×10^{-8} or 4.5×10^{-8} *M*. Control C57BL/6 splenocytes were sham-treated with the vehicle only. Non. sens. means nonsensitized control. Values are expressed as means ± SEM of quadruplicate cultures. *$P < 0.05$, **$P < 0.01$ vs control.

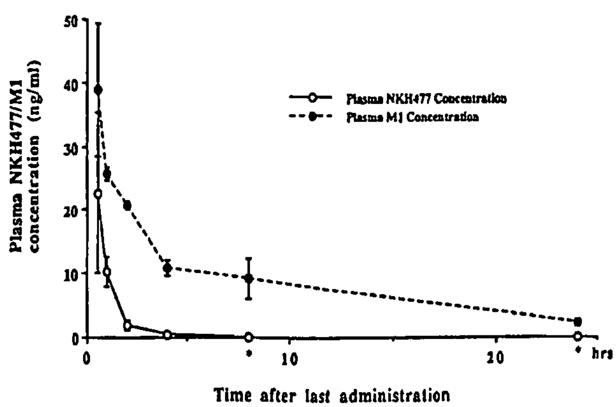

FIG. 4. Pharmacokinetics of NKH477 and its active metabolite M1 in mice. Mice were orally administered NKH477 at 3 mg/kg for 5 consecutive days. Thirty minutes, 1, 2, 4, 8, and 24 hours after last administration, three mice at each point were killed and plasma was collected. Plasma concentration of compounds were measured by gas chromatography/mass spectrometry. Values are expressed as means ± SEM. *Not detectable.

Statistical Analysis

The significance of differences between two nonparametric groups in the allograft survival experiments and scores for histopathological findings were examined by the Mann–Whitney U test. Percentage cytolysis in CTL assay, levels of IL-2 in culture supernatants, and [^{3}H]thymidine incorporation were compared by one-factor ANOVA followed by Fisher's protected least significant difference.

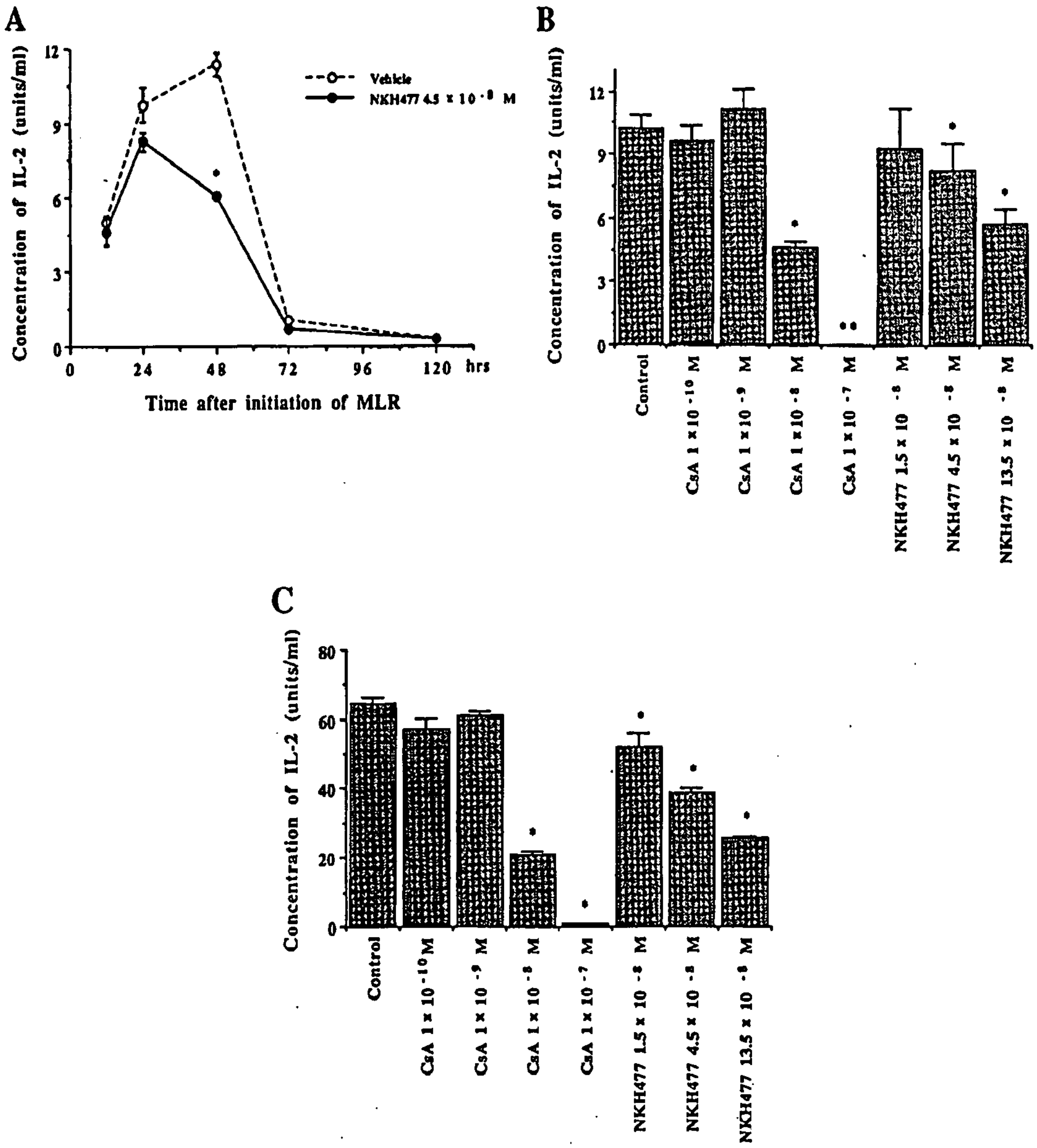

FIG. 6. (A) Serial IL-2 production during allogeneic MLR incubated in the presence of NKH477 or control vehicle. NKH477 was added to the medium at a concentration of 4.5×10^{-8} *M* from the initiation of MLR. Supernatants were collected serially and the concentrations of IL-2 were assayed by ELISA. Values are expressed as means ± SEM of 4 wells. (B) Bar graphs indicate concentrations of IL-2 in the supernatants after 48 hr MLR in the presence of several doses of NKH477 or CsA. NKH477 suppressed IL-2 production during MLR in a dose-dependent manner, although it was less potent than CsA at the concentrations equivalent to the plasma level in clinical use. Values are expressed as means ± SD of 4 wells. (C) Concentrations of IL-2 in the supernatants were quantified after 24 hr mitogen stimulation by 4 μg/ml of Con A in the presence of several doses of NKH477 or CsA. NKH477 and CsA suppressed IL-2 production with dose dependencies similar to the case of MLR. Values are expressed as means ± SD of 4 wells. **P* < 0.01 vs control. **Values were lower than the minimal sensitivity (0.03 units/ml).

RESULTS

Effect of NKH477 and Cyclosporin A on Cardiac Allograft Survival

During the experiments, recipient mice appeared healthy and no differences in body weight were observed among the NKH477-treated, CsA-treated, and saline-treated control groups. In this acute rejection model, saline-treated C57BL/6 recipient mice rejected all DBA/2 cardiac allografts within 12 days with a median survival time of 10 days. Oral administration of a low dose (5 or 10 mg/kg/day) of CsA prolongs graft survival only slightly and 40 mg/kg/day of CsA exhibited only mild but significant effect on graft survival with a median survival time of 15.5 days (Table 1, Fig. 1). In NKH477-treated groups, graft survival was slightly prolonged in mice treated with NKH477 at 1 mg/kg/day to a median survival time of 12 days and mildly prolonged in those treated at 3 mg/kg/day to a median

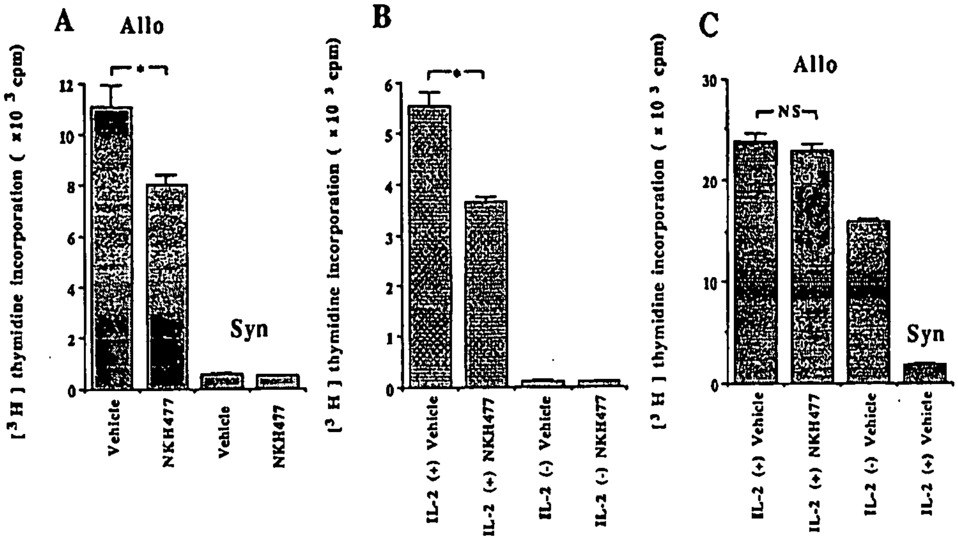

FIG. 7. Effects of NKH477 on [^{3}H]thymidine incorporation by T cell-enriched spleen cells. (A) Splenic T cells from C57BL/6 mice (recipient strain) were cocultured with MMC-treated spleen cells from DBA/2 mice (donor strain, allo) or C57BL/6 (syn). On Day 5 of MLR cells were pulsed for 6 hr with [^{3}H]thymidine (1 μCi/well). (B) Unprimed splenic T cells from the recipient strain were stimulated to proliferate with exogenous IL-2 (1 n*M*). Cells were also cultured without addition of IL-2 to exclude the effect of NKH477 itself on proliferation. (C) Splenic T cells were primed by one-way MLR (allo; allogeneic, syn; syngeneic) and after washing, cells were stimulated with exogenous IL-2. Values are expressed as means ± SEM. *$P < 0.01$.

survival time of 15 days ($P < 0.01$ vs saline-treated control; Mann–Whitney *U* test). However, a low dose of NKH477 (0.3 mg/kg/day) failed to significantly prolong graft survival (median graft survival time 11 days; Table 1, Fig. 2).

Histopathological Examination

As CTL assay confirmed the existence of CTL after MLR for 5 days, histopathological examination was performed in DBA/2-C57BL/6 cardiac allografts harvested 5 days after transplantation. The grafts of the NKH477-treated group (3 mg/kg/day) were compared with those of the saline-treated control group. As shown in Fig. 3, inflammatory changes in the grafts appeared less prominent in the NKH477-treated group. Moreover, the scores for myocardial and perivascular inflammation were significantly lower in the NKH477-treated group ($P < 0.05$). There were no statistically significant differences between the scores for necrosis of the two groups (Table 2).

Pharmacokinetics of NKH477 and Its Active Metabolite M1

Plasma concentrations of NKH477 and M1 were 22.68 ± 12.65 and 39.01 ± 10.46 ng/ml (mean ± SEM), respectively, 30 min after last administration of 3 mg/kg of NKH477. They then declined with half-lives of 0.43 and 1.81 hr, respectively. Twenty-four hours after last administration, no NKH477 and little M1 were detected in the plasma of mice (Fig. 4).

Effect of NKH477 on the Generation of Antigen-Specific CTL

To examine *in vitro* one of the mechanisms of the above findings in allograft survival, we investigated the effect of NKH477 on CTL activity. Specific cytolysis by C57BL/6 splenocytes (recipient strain) of DBA/2 splenocytes (donor strain) and of C3H/He splenocytes (third strain) was measured (Fig. 5). C57BL/6 cells treated with control vehicle and cells treated with 1.5×10^{-8} *M* NKH477 showed similar increases in CTL response to DBA/2 target cells after MLR for 5 days. In contrast, cells treated with 4.5×10^{-8} *M* NKH477 during MLR showed decreased CTL activity. These C57BL/6 cells showed little cytolytic activity when the target cells were from a third strain. Further, nonsensitized C57BL/6 spleen cells also showed little cytolytic activity against DBA/2 target cells.

Effect of NKH477 and CsA on IL-2 Production

In the first experiments, supernatants of allogeneic MLR were harvested serially at 12, 24, 48, 72, or 120 hr after initiation, and concentrations of IL-2 were quantified. As shown in Fig. 6A, addition of NKH477 at a concentration of 4.5×10^{-8} *M* to the culture significantly suppressed IL-2 production at 48 hr after initiation (NKH477 treated vs control vehicle: 6.05 ± 0.13 vs 11.35 ± 0.46; mean ± SEM units/ml; 46.6% suppression) when the control culture exhibited peak production. Figure 6B demonstrates the effects of several concentrations of NKH477 or CsA on the IL-2 production after 48 hr MLR. CsA showed almost complete inhibi-

tion at the equivalent concentration (1×10^{-7} *M*) to the therapeutic plasma level. Addition of NKH477 in the culture medium suppressed IL-2 production dose dependently, although the efficacy of NKH477 was relatively weak in comparison with CsA. NKH477 and CsA also exhibited similar inhibition patterns to the MLR, respectively, on IL-2 production during mitogen response to Con A (Fig. 6C).

Effect of NKH477 on T Cell Proliferation

[^{3}H]Thymidine incorporation was significantly inhibited by NKH477 at a concentration of 4.5×10^{-8} *M* (80,056 ± 3656, mean cpm ± SEM) compared with the control vehicle (111,175 ± 8282) in allogeneic MLR with a percentage inhibition of 28.0% and in IL-2 stimulation of unprimed splenic T cells (NKH477 vs control vehicle: 36,463 ± 1151 vs 55,565 ± 2694) with a percentage inhibition of 34.4% (Figs. 7A and 7B). However, the drug at the same concentration could not significantly suppress the proliferative response to exogenous IL-2 of cells primed and activated by allogeneic MLR (NKH477 vs control vehicle: 229,344 ± 6381 vs 238,636 ± 7966) (Fig. 7C).

DISCUSSION

We investigated the effects of NKH477 on cardiac allograft survival and on histopathological findings in the early stage of acute cellular rejection. In this acute rejection model, all DBA/2 cardiac allografts in saline-treated C57BL/6 recipients without any immunosuppression were rejected within 12 days. Lower doses of CsA (5 or 10 mg/kg/day) had only slight effect on graft survival and 40 mg/kg/day of CsA had mild effect in this model. Considering body surface area, the oral dose of 40 mg/kg/day in mice is comparable to 3 mg/kg/day in human (27) and this dose is the maintenance dose of CsA in clinical cardiac transplantation. Treatment with NKH477 at 1 mg/kg/day beginning on the day of transplantation resulted in slight prolongation of allograft survival, and at 3 mg/kg/day in a mild prolongation of survival with a similar median survival time to that of the maintenance dose of CsA (Table 1). Inflammatory changes were less prominent in animals treated with NKH477 at 3 mg/kg/day than in the vehicle-treated group. Although no significant difference between the two groups was seen in the score for necrosis, it seems to be the reason that necrosis becomes apparent later in the course of rejection.

cAMP-elevating agents such as forskolin have been suggested to have immunosuppressive effects on lymphocytes. For example, they have an inhibitory effect on IL-2-secreting Th1 lymphocytes *in vitro* (1), and decrease IL-2 production and IL-2Rα (IL-2 receptor α-chain) expression via the suppression of both protein product and mRNA levels (2). Blockade of the IL-2 pathway suppresses immunological responses to alloantigens and prevents acute allograft rejection (28–30). The increase in [cAMP]$_i$ induced by forskolin and other agents is related to the inhibition of T cell proliferation (1, 31, 32). It also leads to the inhibition of CTL activity (4, 5). While CTL, activated and induced to proliferate after stimulation by alloantigens, are considered to play an important role in the course of allograft rejection (7–10), CTL generated in the presence of forskolin showed suppressed lytic activity but normal proliferative responses to alloantigens (5).

Considering these reports and the results of *in vivo* experiments, we tried to clarify the effects of NKH477 on alloreactive CTL generation and IL-2 production during MLR and mitogen response to Con A. We compared its inhibitory effect on IL-2 production to that of a standard immunosuppressant CsA, since CsA is considered to exhibit immunosuppressive effects mostly via inhibition of IL-2 gene transcription.(33–35) We used a concentration of NKH477 equivalent to the plasma level in *in vivo* experiment (Fig. 4). NKH477 at 4.5×10^{-8} *M* (24.6 ng/ml) inhibited the generation of alloreactive CTL and suppressed IL-2 production during MLR and mitogen response; however, it was less effective than equivalent concentration of CsA to the therapeutic plasma level. Prolongation of cardiac allograft survival might have resulted from this suppression of IL-2 production, reflecting elevated expression of cytokines such as IL-2 in organ allografts undergoing acute rejection (36). These results also suggest some possible mechanisms by which NKH477 may have inhibited the generation of CTL. NKH477 might first suppress IL-2 production, and the resulting decrease in IL-2 concentration may inhibit the differentiation of CTL. NKH477 might inhibit T cell proliferation through the decreased production of IL-2 or through the reduced responsiveness to IL-2 of T cells (including CTL precursor), thereby leading to inhibition of CTL generation, although other humoral factors for CTL generation have not been investigated in this study. We therefore assessed whether NKH477 had inhibitory effects on T cell proliferation during MLR and on the proliferative responses to IL-2 of primed and unprimed splenic T cells using [^{3}H]thymidine incorporation. The results indicated that NKH477 had an inhibitory effect on T cell proliferative response when present in the culture medium throughout MLR or when unprimed T cells were stimulated with IL-2 in the presence of this drug. However, it did not have an inhibitory effect on IL-2 responsiveness of alloantigen-primed T cells at its physiological dose. These results indicate that NKH477, a cAMP-elevating agent, cannot block the progression of alloantigen-primed T cell proliferation at its physiological dose, although the possibility remains that it may counteract the initiation of T cell proliferation.

The combined concentration of NKH477 and its equally active metabolite M1 in the plasma were kept

higher than 4.5×10^{-8} *M*, the concentration in the culture medium in *in vitro* experiments, at 4 hr after the last administration. This suggests that immunosuppressive effects of NKH477 in the *in vivo* experiments were exerted through modulation of T cell functions such as inhibition of CTL generation, while the drug could not have a suppressive effect on the proliferative response of primed T cells and the effects might not be specific to the suppression of allo-reactive immunity.

Valitutti *et al.* reported that increases in $[cAMP]_i$ suppressed the adhesion and motility of CTL, possibly by regulation of elements of their cytoskeleton formation such as the F-actin and tubulin network (6). Thus adenylate cyclase activators inhibit functions of generated CTL. Improved histopathological findings in the early stages of NKH477-treated allografts are also suggestive of these effects. In addition, recent studies have shown that cyclic AMP analogs and cAMP-elevating agents enhance cardiac preservation in rat and baboon cardiac transplant models through their beneficial effect on vascular homeostatic mechanisms during global ischemia (37, 38). NKH477 may improve oxygen supply to ischemic regions of allografts undergoing rejection by maintaining vascular functions(possibly endothelial function) (37), thereby prolonging graft survival. Another beneficial effect of NKH477 on vascular function is its vasodilating effect in coronary arteries. In smooth muscle cells of coronary arteries, NKH477 attenuates acetylcholine-induced Ca^{2+} mobilization and reduces the sensitivity of contractile machinery to Ca^{2+}, possibly by activating cAMP-dependent mechanisms (39).

In conclusion, NKH477, a water-soluble forskolin derivative, has inhibitory effects on antigen-specific CTL activities, and other beneficial effects on cardiac allograft survival. Concerning inhibitory effects on IL-2 production, NKH477 was less effective than CsA and the drug might have less efficacy on *in vivo* immunosuppression than moderate to high doses of CsA. However, considering that NKH477 also has a positive inotropic effect on failing hearts, this drug may be a promising agent for the management of hemodynamic states of patients after cardiac transplantation and for therapy against the cardiac dysfunction which emerges as a manifestation of acute rejection. Further studies are required to assess the beneficial effects of this drug and roles of immunomodulation by inotropic agents.

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RAPID COMMUNICATION

Combination Therapy with a CD4-CDR3 Peptide Analog and Cyclosporin A to Prevent Graft-vs-Host Disease in a MHC-Haploidentical Bone Marrow Transplantation Model[1]

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Graft-versus-host disease (GVHD) is a major complication associated with allogeneic bone marrow transplantation (BMT). Cyclosporin A (CsA) has been used as the basis for most immunosuppressive regimens for the prevention of GVHD, but has exhibited only limited effects and is hampered by nephrotoxicity, neurotoxicity, and hepatotoxicity. Previously, we showed that rD-mPGPtide, a structure-base designed peptide analog of the CDR3-like region of domain 1 of the murine CD4 molecule, suppressed the development of GVHD in a MHC-haploidentical murine BMT model when administered early in the course of disease. This peptide analog also inhibited T cell proliferation in mixed lymphocyte reactions (MLR) *in vitro.* The current results demonstrate that CsA and rD-mPGPtide exhibit an additive inhibitory effect on MLR. Furthermore, the use of CsA and rD-mPGPtide together for prevention of GVHD nearly doubled the median survival time of the mice compared to either agent alone. In addition, the combination therapy reduced the requirement for habitual administration of CsA. Therefore, the use of a CD4-CDR3 peptide can complement and potentiate the immunosuppressive effects of CsA in the prevention of GVHD following allogeneic BMT.

Key Words: **rD-mPGPtide; CD4 peptide; cyclosporin A; graft-vs-host disease; bone marrow transplantation.**

INTRODUCTION

Allogeneic bone marrow transplantation (BMT) is currently being used as a treatment for a number of disease states including leukemias, multiple myeloma myelodysplasia, aplastic anemia, and severe combined immunodeficiency (1). The major complications which impede the overall success of this treatment include the development of graft-versus-host disease (GVHD), failure to engraft, infections resulting from chronic immunoincompetence, and leukemic relapse. Acute GVHD is caused by contaminating mature donor T cells in the bone marrow graft and leads to significant morbidity and mortality. The inhibition of T cell responses following BMT, by various means, has proven to be effective in preventing GVHD. Cyclosporin A (CsA), which has moderate effects on GVHD, has become the basis for many of the immunosuppressive regimens (1).

CsA has been shown to be a potent immunosuppressive agent for solid organ transplantation and is currently being tested for treatment of several autoimmune diseases (2). CsA is thought to specifically inhibit lymphocytes by binding to cyclophilin (CyP), and this CsA–CyP complex binds to and blocks the function of calcineurin. Calcineurin, a phosphatase, plays a critical role in the signaling cascade of T cells leading to the production of cytokines such as interleukin-2 (IL-2) (5, 6). Although CsA has been successfully used for the treatment of GVHD following allogeneic BMT, it has associated drawbacks, including hepatotoxicity, neurotoxicity, and nephrotoxicity (1, 2). Previous studies have also indicated that CsA can interfere with the normal thymic development of T cells leading to immunoincompetency and the potential generation of autoreactive T cells (3). Equally important is the need to administer frequent doses of CsA to patients for up to 6 months or more post-BMT, since alloreactive donor T cells can survive treatment and become activated once immunosuppression is halted (1).

Our earlier reports documented that the CD4-CDR3 peptide analog, rD-mPGPtide, was a potent inhibitor of murine CD4 T-cell-mediated immune responses, both *in vitro* and *in vivo* (4, 7, 8). In this regard, we also demonstrated that rD-mPGPtide suppressed the *in vivo* alloreactive responses associated with the onset of GVHD in the (B6 × DBA2)F_1 → (B6 × CBA)F_1 MHC-haploidentical irradiated BMT model (8). In this model, the donor and recipient mice possess a full MHC mismatch, involving both class I and class II differences.

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Injection of irradiated (950 cGy) (B6 × CBA)F$_1$ mice with an inoculum of donor T cells along with donor bone marrow induced an acute GVHD reaction which resulted in fatality within 2–3 weeks. The current study indicated that rD-mPGPtide and CsA could inhibit T cell responses *in vitro* and were more potent together than either agent was alone. Furthermore, administration of rD-mPGPtide to recipient mice following BMT significantly increased the median survival time (MST) of mice with GVHD, as did the administration of CsA. However, when rD-mPGPtide and CsA were administered together, survival was greater than when either agent was administered alone.

MATERIALS AND METHODS

Mice. Mice, (B6 × DBA2)F$_1$ [(B6D2)F$_1$] (H-2^{bd}) and (B6 × CBA2)F$_1$ [(B6CBA)F$_1$] (H-2^{bk}), were purchased from The Jackson Laboratory (Bar Harbor, ME). Male mice were used as donors between the ages of 7 and 12 weeks and as recipients between the ages of 9 and 16 weeks. Mice were kept in a sterile environment in microisolators at all times and were provided with acidified water and autoclaved food.

Media. Buffered saline solution (BSS) supplemented with 0.1% BSA (Hyclone, Logan, UT) was used for all *in vitro* manipulations of the donor bone marrow and lymphocytes. For injection, cells were resuspended in BSS alone. RPMI 1640 (Mediatek, Herndon, VA) supplemented with 10% FCS (Sigma, St. Louis, MO) and 10 U/ml glutamine, 10 U/ml penicillin and streptomycin, and 0.05mM BME (Mediatech, Inc. Herndon, VA) was used for all *in vitro* mixed lymphocyte responses.

Peptides. The peptides were designed as previously described (7), synthesized on an Applied Biosystems, Inc. (Foster City, CA) 430A peptide synthesizer using standard Fmoc chemistry, refolded to enrich for intramolecular disulfide bonding, and purified by HPLC (Waters 600E system controller, Waters 490E programmable multiwavelength detector, Millipore Corp, Bedford, MA) before use. The sequences of the synthesized peptides were as follows: rD-mPGPtide (CPGPEEKRNELEC, all D-amino acids) and scrambled rD-mPGPtide (Scr-PGPtide; same amino acid composition, but scrambled sequence, CEPKNELPERGEC, all D-amino acids). For treatment of GVHD, peptides were reconstituted in BBS and injected at the appropriate dose and time into mice iv in a volume of 0.25 ml.

Injections. All cell suspensions were given intravenously via the tail vein in a maximum volume of 0.5ml of BSS.

Irradiation. All recipient mice received a 950-cGy exposure from a Gammacell ^{137}Cs source (116 cGy/min).

mAbs. Ascites fluid for anti-Thy-1.2 (J1j, rat IgM) and anti-CD8 (3.168, rat IgM) mAb were used for cell preparations. In addition, goat anti-mouse IgG (whole molecule) antibodies were purchased from Cappel-Organon Teknika (Westchester, PA). Guinea pig serum prepared in our laboratory was used as a source of complement C for all mAb treatments.

Preparation of cells. Bone marrow cells were obtained from the femurae and tibiae of donor mice by flushing with BSS w/0.1% BSA. To prepare anti-Thy-1-treated (T cell depleted) bone marrow (ATBM), cells were incubated with J1j mAb (at 1:100 dilution) and C (1:25) for 45 min at 37°C and were washed four times. T-cell-enriched donor cell populations were prepared by treating pooled spleen and lymph node cells with: (a) Gey's balanced salt lysing solution containing 0.7% NH_4Cl for removal of RBC; and (b) panning on a plastic petri dish precoated with a 5 μg/ml solution of goat anti-mouse IgG for 1 h at 37°C to remove B cells. These treatments resulted in populations of 90–95% CD3$^+$ cells, as quantitated by flow cytometric analysis. Further purification of T cells into CD4$^+$ cells was performed as described previously. These procedures resulted in purified populations of CD4$^+$ cells (>90%) with no detectable presence of the inappropriate subset.

Mortality assay for GVHD. Recipient mice were irradiated with 950 cGy and approximately 6 h later were injected intravenously with either donor ATBM (2 × 10^6) alone as a negative control or a mixture of ATBM plus donor T cells as indicated. Mice were checked daily for morbidity and mortality until the experiments were terminated at day 60 after transplantation. MST were calculated as previously described. Statistical comparisons between experimental groups for mortality curves were performed by the nonparametric Wilcoxon signed rank analysis.

RESULTS

The effect of rD-mPGPtide and cyclosporin A on alloreactivity in vitro. To assess the effect of combining rD-mPGPtide and CsA on T cell responses *in vitro*, lymph node cells taken from (B6 × DBA2)F$_1$ mice were stimulated *in vitro* with irradiated (15 Gy) spleen cells from (B6 × CBA)F$_1$ mice. The proliferative response was measured by pulsing with [^{3}H]TdR for the final day of a 5-day culture. Titered concentrations of rD-mPGPtide (50–200 μM) were added to appropriate culture wells in order to test the inhibitory properties of the peptide. As indicated in Table 1a, rD-mPGPtide inhibited proliferation in a dose dependent manner. Nearly 90% inhibition of the proliferative response was observed with the addition of 200 μM rD-mPGPtide, and the 50% inhibitory concentration (IC$_{50}$) was approximately 100 μM. CsA was also a potent inhibitor

TABLE 1

In Vitro Inhibition of MLR by a Combination of rD-mPGPtide with CsA

rD-mPGPtide (μM)	CsA (μg/ml) 0	0.001	0.01	0.1
(A) (B6xDBA2)F_1 Anti-(B6xCBA)F_1				
0	31.5 ± 3.3	23.1 ± 1.2	7.5 ± 3.6	ND
50	24.8 ± 13.6	19.5 ± 0.8	9.0 ± 9.1	ND
100	15.6 ± 4.7	12.9 ± 5.3	3.5 ± 1.0	ND
200	4.2 ± 0.8	2.7 ± 1.2	2.7 ± 0.5	ND
(B) CBA Anti-C57BL/6				
0	14.1 ± 3.0	ND	9.7 ± 0.5	4.2 ± 1.4
17	7.7 ± 1.8	ND	6.0 ± 1.1	2.1 ± 0.7
33	5.9 ± 2.7	ND	3.6 ± 0.9	2.8 ± 0.6
67	2.5 ± 1.4	ND	1.5 ± 0.3	1.0 ± 0.7

Note. Data are expressed as a response index ± SD. Response index is defined as the ratio of the [^{3}H]TdR incorporated by experimental cells and the [^{3}H]TdR incorporated by unstimulated responder cells. ND, no data. In each well, 2×10^5 responder cells were stimulated with 4×10^5 irradiated stimulator cells. Wells were pulsed with 1 μCi [^{3}H]TdR.

of the MLR in the range of 1–10 ng/ml. In addition, the peptide and CsA exhibited an additive effect on inhibiting the proliferative response of the MLR, i.e., suppression was greater when the reagents were added together than with either one alone. This additive effect was also observed in MLR when other strain combinations were utilized (Table 1b).

The effect of combined treatment of rD-mPGPtide and cyclosporin A on GVHD. In an attempt to evaluate the effect of combining rD-mPGPtide and CsA *in vivo*, an acute form of GVHD was induced in lethally irradiated (9.5 Gy) (B6 × CBA)F_1 mice by administration of 5×10^6 (B6 × DBA2)F_1 T cells along with 2×10^6 (B6 × DBA2)F_1 ATBM. In this GVHD model, the untreated mice exhibited 80% fatality by day 20 posttransplant with a MST of 13 days (Fig. 1A). Experimental groups received either a daily ip injection of CsA at a suboptimal dose of 10 mg/kg, or rD-mPGPtide (0.5 mg iv) on days 0, 3, and 6. The MST for mice treated with either rD-mPGPtide or CsA was 28 and 30 days, respectively. A combination of the two treatment regimens resulted in significant prolongation of survival (MST of 53 days) compared to the untreated group ($P < 0.05$). In addition, the survival of the combination treatment group was significantly prolonged in comparison to either treatment alone ($P \leq 0.04$). Thus, rD-mPGPtide and CsA have an additive inhibitory effect on the development of GVHD across MHC barriers.

Combination of rD-mPGPtide and short-term CsA prophylaxis for GVHD. The inherent toxicity and persistent immunosuppression associated with CsA are major complications associated with long-term administration for the prevention of GVHD. In this regard, it was hypothesized that rD-mPGPtide could eliminate the need for long-term CsA administration. To test this notion in the same haploidentical BMT model as described above, GVHD was induced by the transplantation of 5×10^6 donor (B6 × DBA2)F_1 T cells, and mice were either left untreated, injected iv with rD-mPGPtide (0.5 mg, days 0, 3, 6), or administered CsA (20 mg/kg) ip daily for 8 weeks (long-term CsA; Fig. 1B). Two additional experimental groups received CsA either alone (20 mg/kg) ip daily for only 2 weeks (short-term CsA) or in combination with rD-mPGPtide (0.5 mg iv, days 0, 3, 6). The survival of the rD-mPGPtide-treated mice (MST of 18 days) was significantly enhanced ($P \leq 0.01$) compared with that of the untreated group (MST of 8 days). Both CsA-treated groups exhibited enhanced survival compared with the untreated group as well ($P \leq 0.01$). However, short-term CsA administration (MST of 43 days) was less effective than long-term CsA administration (MST of 61 days). The coadministration of rD-mPGPtide with short-term CsA therapy (MST of 59 days) significantly enhanced survival of these mice ($P \leq 0.04$) compared with the short-term CsA alone. It should also be noted that the survival of the combined rD-mPGPtide and short-term CsA-treated mice was equivalent to that achieved with long-term CsA administration ($P = 0.75$).

DISCUSSION

It is apparent from our results that administration of rD-mPGPtide and CsA together can significantly inhibit T cell reactivity both *in vitro* and *in vivo*, compared with either agent alone. The additive effect these agents display would allow for the reduced utilization of the more toxic immunosuppressive compound (CsA) while still maintaining the high degree of recipient sur-

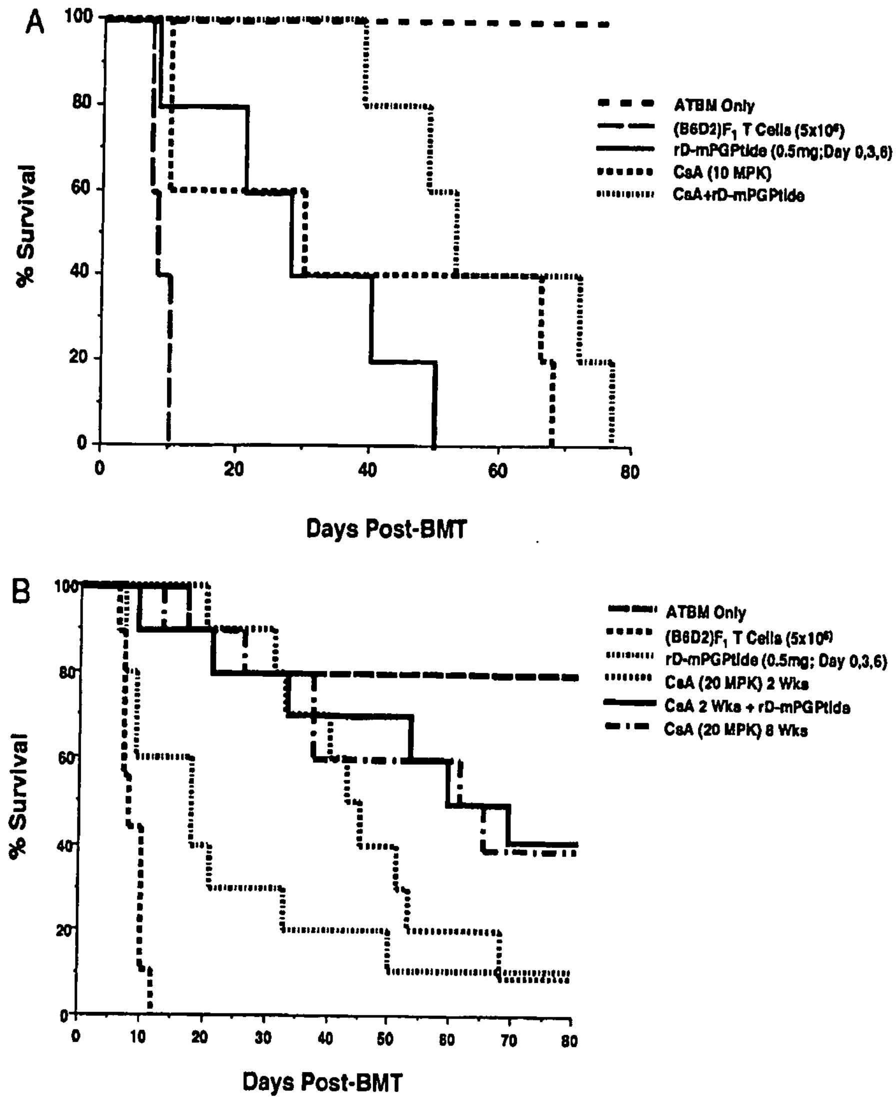

FIG. 1. Effect of treatment with rD-mPGPtide and CsA on survival of mice undergoing GVHD across a MHC-haploidentical barrier. (B6 × CBA)F_1 mice were lethally irradiated (950 cGy) and transplanted with allogeneic (B6 × DBA2)F_1 ATBM (2 × 10^6) cells alone, or with unseparated donor T cells (5 × 10^6) as indicated. (A) Recipients of T cells were either left untreated (positive GVHD control), injected iv with rD-mPGPtide (0.5 mg) every third day between days 0 and 6, injected ip with CsA (10 mg/Kg) daily, or given both regimens as indicated in the figure. The data are representative of three separate experiments and 5 mice were utilized per group. (B) Recipients of T cells were either left untreated (positive GVHD control), injected iv with rD-mPGPtide (0.5 mg) every third day between days 0 and 6, injected ip with CsA (10 mg/Kg) daily for 2 or 8 weeks, or given combinations of both regimens as indicated in the figure. The data are composites from two separate experiments, a total of 10 mice were utilized per group and significance was determined by non-parametric Wilcoxon signed rank analysis utilizing SYSTAT 5.2 software.

vival following BMT seen with continuous utilization of CsA. The probable differences in the mechanisms of action of these compounds allow for the speculation that these two agents could suppress T cells in a complimentary manner.

The mechanism by which CsA inhibits T cell responses via inhibition of IL-2 production is well known and can be partially blocked by the addition of exogenous IL-2. While the exact mechanism of rD-mPGPtide-mediated immunosuppression is still unclear, we previously demonstrated that the inhibition induced by rD-mPGPtide is not overcome by the addition of exogenous IL-2 (8), thus making the combined immunosuppressive effect more resistant to circumvention by cytokine rescue. Furthermore, rD-mPGPtide is an analog of the murine CD4 molecule and has demonstrated little activity against $CD8^+$ T cell responses. CsA is not specific to either T cell subset and thus will suppress $CD8^+$ T cell responses. Thus, combining CsA with the peptide allows for inhibition of both T cell subsets which contribute to GVHD. We have previously postulated the rD-mPGPtide acts on T cells by disrupting the formation of the multimeric

CD4 complexes formed on the surface of the T cell upon TCR ligation thus preventing the proper cosignaling required for fulminant activation. Since it has been demonstrated that CsA inhibits the TCR signaling pathway by binding to calcineurin, the combination of the two agents would disrupt both the primary and secondary signaling mechanisms required for T cell activation. The result of inhibiting two of the major signal pathways simultaneously could result in the enhanced immunosuppressive activity seen.

The data thus far suggest only an additive effect of these two agents on T cell responses and more experimentation is required to demonstrate a synergistic effect of the compounds. In any case, the CD4-CDR3 peptide is indeed compatible with CsA treatment and in fact enhances its efficacy in preventing the development of GVHD following allogeneic BMT.

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Inhibitory Effect of a CD4-CDR3 Peptide Analog on Graft-Versus-Host Disease Across a Major Histocompatibility Complex-Haploidentical Barrier

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A structure-based designed peptide has been engineered to exhibit the same molecular surface as a portion of the CDR3-like region in domain 1 of the murine CD4 molecule. Earlier in vitro experiments indicated that this analog, known as rD-mPGPtide, inhibited T-cell proliferation in mixed lymphocyte reactions and blocked activation of both normal CD4⁺ T cells and T-cell lines after T-cell receptor triggering. In addition, rD-mPGPtide proved to be a potent inhibitor in vivo of CD4⁺ T-cell–mediated experimental allergic encephalomyelitis disease in the SJL mouse model. In this current report, we have evaluated the potential of rD-mPGPtide for suppressing the development of graft-versus-host disease (GVHD) in an irradiated major histocompatibility complex (MHC)-haploidentical murine bone marrow transplantation (BMT) model [(B6 × DBA/2)F₁ → (B6 × CBA)F₁ (950 cGy)]. Our results indicated that early administration of rD-mPGPtide was effective in the inhibition of alloreactive responses of the donor T cells against the host and thus delayed or prevented the onset of GVHD. The median survival time of animals treated with rD-mPGPtide was enhanced as much as four-fold with as little as a single dose of peptide at the time of transplant. Decreased alloreactivity was indicated by phenotypic and functional analysis of positively selected thoracic duct lymphocytes 4 days after transplant and by histopathological examination of skin and gastrointestinal tissue samples 4 weeks later. Therefore, the administration of a CD4-CDR3 peptide is an efficacious approach against the development of GVHD during allogeneic BMT.

ALLOGENEIC BONE MARROW transplantation (BMT) is currently being used as a treatment for a number of disease states including several types of leukemia, aplastic anemia, and severe combined immunodeficiency, among others.[1] The major complications that impede the overall success of this treatment include the development of graft-versus-host disease (GVHD), marrow graft rejection, chronic immuno-incompetence, and leukemic relapse (in the case of BMT for the treatment of leukemias). Acute and chronic GVHD is caused by residual mature donor T cells in the bone marrow graft and leads to significant morbidity and mortality.[2] Removal of the mature T cells from the graft before engraftment reduces or prevents GVHD; however, this T-cell depletion also leads to reduced engraftment along with increased leukemia relapse rates.[3-5] These observations suggest the importance of a T-cell component in a successful BMT, although it is not clear whether GVHD reactive T cells can be separated completely from either the antileukemia effect or from enhanced hematopoietic engraftment. This question can only be approached by highly selective means of inhibiting those host-allospecific GVHD-reactive T cells, while allowing for the potential development of antileukemia-specific responses and for protection from opportunistic infections.

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The CD4 molecule on the surface of helper T cells, in association with the T-cell receptor (TCR)-CD3 complex that recognizes specific antigen in the context of MHC class II, plays a critical role in the transmembrane and intracellular signaling pathways required for T-cell activation.[6-11] It has been well-established in murine models that CD4⁺ T cells are capable of mediating GVHD, primarily across MHC class II barriers,[12,13] but also in some cases with minor histocompatibility antigenic differences.[14,15] Inhibiting CD4⁺ T-cell responses by treatment of recipient mice with monoclonal antibodies (MoAb) directed against the CD4 molecule has effectively decreased the incidence of GVHD following BMT.[16-18] However, MoAb therapy has several limitations for potential clinical use, including but not limited to total subset depletion and immunogenicity of the MoAb itself.[19-21]

In previous reports, we have described the design and production of a peptide that specifically mimics the CDR3-like region in the D1 immunoglobulin domain of the murine CD4 molecule.[22,23] This peptide analog consists of thirteen amino acids (CELENRKEEPGPC) taken from the p86-94 sequence of the CD4 molecule with the addition of a proline-glycine-proline-cysteine sequence to the carboxyl terminus to allow cyclization and tertiary structural constraint. In order to make the peptide more resistant to protease degradation, it was synthesized with D-amino residues, necessitating the reversal of the amino acid order so that side chain presentation would be similar to the native molecule.[23] As a result of these adaptations, this CD4 peptide analog is referred to as reverse D amino acid mouse proline-glycine-proline peptide (rD-mPGPtide). The rD-mPGPtide is neither T-cell subset depletive nor immunogenic and thus has advantages over the use of anti-CD4 MoAb.

Our earlier reports documented that rD-mPGPtide is a potent inhibitor of certain types of CD4⁺ T-cell–mediated immune responses both in vitro and in vivo. In this report, we demonstrate the potential of rD-mPGPtide for inhibiting the in vivo alloreactive responses associated with the onset of GVHD in a major histocompatibility complex (MHC) haploidentical murine BMT model (B6 × DBA/2)F_1 → (B6 × CBA)F_1 (950 cGy). In this model, the donor and recipient mice possess both class I and class II differences. Injection

of irradiated (B6 × CBA)F_1 mice with a donor bone marrow inoculum supplemented with either 5×10^6 unseparated or 1×10^6 $CD4^+$ enriched donor T cells induces an acute form of GVHD, which leads to fatality within 2 to 3 weeks. We show here that administration of rD-mPGPtide to recipient mice at varying times within the first week of transplantation significantly increased the median survival time of mice undergoing GVHD.

MATERIALS AND METHODS

Mice. Mice, (B6 × DBA/2)F_1, [(B6D2)F_1 ($H2^{b/d}$)] and (B6 × CBA)F_1 [(B6CB)F_1 ($H2^{b/k}$)], were purchased from the Jackson Laboratory (Bar Harbor, ME). Male mice were used as donors between the ages of 7 to 12 weeks and as recipients between the ages of 9 to 16 weeks. Mice were kept in a sterile environment in microisolators at all times and were provided with acidified water and autoclaved food.

Media. Buffered saline solution (BSS) supplemented with 0.1% bovine serum albumin (BSA) (Hyclone, Logan, UT) was used for all in vitro manipulations of the donor bone marrow and lymphocytes. For injection, cells were resuspended in BSS alone. RPMI 1640 (Mediatek, Herndon, VA) supplemented with 10% fetal calf serum (FCS) (Sigma, St Louis, MO) and 10 U/mL glutamine, 10 U/mL penicillin and streptomycin, and 0.05 mmol/L β-mercaptoethanol (Mediatech) was used for all in vitro mixed lymphocyte responses.

Peptides. The peptides were designed as previously described,[24] synthesized on an Applied Biosystems 430A peptide synthesizer (Foster City, CA) using standard Fmoc chemistry, refolded to enrich for intramolecular disulfide bonding, and purified by HPLC (Waters 600E system controller, Waters 490E programmable multi-wavelength detector; Millipore Corp, Bedford, MA) before use. The sequences of the synthesized peptides were as follows: rD-mPGPtide (CPGPEEKRNELEC, all D-amino acids) and scrambled rD-mPGPtide (Scr-PGPtide; CEPKNELPERGEC, all D-amino acids). For treatment of GVHD, peptides were reconstituted in PBS and injected at the appropriate dose and time into mice intravenously (IV) in a volume of 0.25 mL.

Irradiation. All recipient mice received a 950 cGy exposure from a Gammacell ^{137}Cs source (116 cGy/min).

MoAb. Ascites fluid for anti–Thy-1.2 (J1j, rat IgM)[25] and anti-CD8 (3.168, rat IgM)[26] MoAb were used for cell preparations. In addition, goat antimouse IgG (whole molecule) antibodies were purchased from Cappel-Organon Teknika (Westchester, PA). Guinea pig serum prepared in our laboratory was used as a source of C for all MoAb treatments. For phenotypic analysis of cells by flow cytometry, anti-murine CD4 (FITC-conjugated or biotinylated, as appropriate), CD25, CD71, CD95 (all biotinylated), and rat IgG (FITC- or PE-conjugated, as appropriate) standard control antibodies were purchased from Pharmingen (San Diego, CA).

Preparation of cells. Bone marrow cells were obtained from the femora and tibiae of donor mice by flushing with BSS with 0.1% BSA. To prepare anti–Thy-1–treated (T-cell–depleted) bone marrow (ATBM), cells were incubated with J1j MoAb (at 1:100 dilution) and C (1:25) for 45 minutes at 37°C and were washed four times. T-cell enriched donor cell populations were prepared by treating pooled spleen and lymph node (LN) cells with: Gey's balanced salt lysing solution containing 0.7% NH_4Cl for removal of RBC, and panning on a plastic petri dish pre-coated with a 5 μg/mL solution of goat antimouse IgG for 1 hour at 37°C to remove B cells. These treatments resulted in populations of 90% to 95% $CD3^+$ cells, as quantitated by flow cytometric analysis. Further purification of T cells into $CD4^+$ cells was performed as described previously.[14] These procedures resulted in highly purified populations of $CD4^+$ cells (>90%) with no detectable presence of the inappropriate subset.

Flow cytometric analysis. In a 96-well plate, 2×10^5 cells/sample were incubated and washed with BSS containing 1% fetal bovine serum (FBS) and 0.05% NaN_3 (FACS buffer). Antibodies, conjugated to either FITC or biotin, were added to the appropriate wells in a volume of 25 μL for 30 minutes at 4°C then washed three times in FACS buffer and fixed overnight at 4°C in PBS containing 1% paraformaldehyde. In the case of the biotin conjugated antibodies, PE-streptavidin (Caltag, San Francisco, CA) was added (1:100 dilution) before fixation with paraformaldehyde and incubated for an additional 30 minutes at 4°C, then washed three times in FACS buffer and fixed with paraformaldehyde. Samples were analyzed on a Coulter Epics Profile II (Coulter Corp, Hialeah, FL).

In vitro mixed lymphocyte reaction (MLR). Single cell suspensions of responder cells for the murine MLR were obtained from either spleen and lymph nodes or TDL, as indicated. Stimulator cells were obtained from the spleens of indicated mice, irradiated with 15 Gy and washed three times with medium. In a 96-well plate 4×10^5 responder cells were incubated with 8×10^5 stimulator cells or medium alone for the indicated period of time at 37°C, 5% CO_2. Cultures were incubated with 1 μCi [^{3}H]TdR/well for the final 24 hours, harvested, and counted. The percent response was calculated in the following manner: experimental CPM [^{3}H]TdR minus medium alone CPM)/(anti-(B6CB)F_1 only CPM [^{3}H]TdR minus medium alone CPM). Responses indices were calculated as a ratio of the experimental CPM [^{3}H]TdR to the anti-(B6D2)F_1 only CPM [^{3}H]-TdR. When indicated, culture supernatants were removed and tested for cytokine production by CTLL bioassay, as previously described.[27] Briefly, 1×10^4 CTLL cells in 25 μL medium were added to 100 μL of culture supernatant in a 96-well plate. Anti-murine interleukin-2 (IL-2) MoAb was added at 2 μg/mL in 25 μL to appropriate wells. Cells were cultured for 24 hours and incubated with 1 μCi [^{3}H]TdR/well for the final 6 hours, harvested, and counted. Experimental results were compared with a standard curve of mIL-2 and IL-4. Statistical comparisons between experimental groups for proliferation responses were performed by the Student's *t*-test analysis, using SYSTAT 5.2 software.

Collection of thoracic duct lymphocytes (TDL). Anesthetized mice were cannulated 4 days after injection of 10^7 (B6D2)F_1 $CD4^+$ T cells by insertion of an Intramedic PE 50 tubule into a fistula perforated in the cysterna chylae, as previously described.[28] The mice were then placed on an apparatus which allows the mice exercise and access to food while they are being infused IV with physiologic saline. The lymph was collected for 8 to 10 hours in 15-mL tubes containing 2 mL RPMI 1640 medium supplemented with 10% FBS, 1 U/mL heparin, and kept at 4°C until assay.

Mortality assay for GVHD. Recipient mice were irradiated with 950 cGy and approximately 6 hours later were injected IV (in a maximum volume of 0.5 mL of BSS) with either 2×10^6 donor ATBM cells alone, as a negative control, or a mixture of ATBM plus donor T cells, as indicated. Mice were checked daily for morbidity and mortality until the experiments were terminated at day 60 posttransplantation. Median survival times (MST) were calculated as previously described.[14] Statistical comparisons between experimental groups for mortality curves were performed by the nonparametric Wilcoxon signed rank analysis, using SYSTAT 5.2 software.

Histopathological analysis. Two mice per experimental group were killed on day 27 post-BMT and organs were removed and fixed with 4% paraformaldehyde. Ear skin and gut were then processed for embedding in paraffin. Paraffin sections (6 μ) were cut and stained with hematoxylin and eosin (H&E). Sections were examined microscopically as indicated for the presence of inflammatory infiltrates and dyskeratotic or necrotic cells.

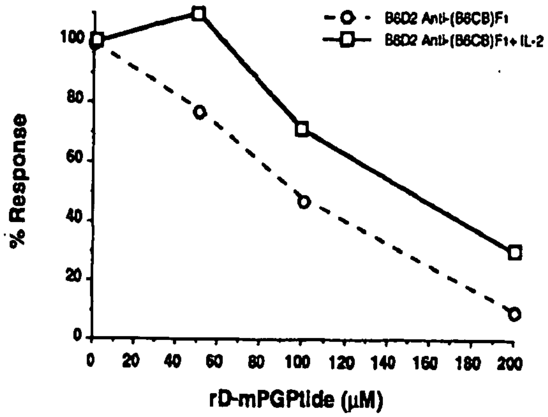

Fig 1. Inhibition of in vitro allogeneic MLR by rD-mPGPtide. The data shown are representative of three separate experiments and expressed as mean percent response [^{3}H]TdR incorporation of triplicate wells. The rD-mPGPtide was added at 200, 100, and 50 μmol/L to the appropriate wells and rhIL-2 was added at 100 U/mL to the appropriate wells.

RESULTS

rD-mPGPtide inhibits alloreactivity in vitro. Lymph node cells taken from (B6D2)F_1 mice were stimulated in vitro with irradiated (15 Gy) spleen cells from (B6CB)F_1 mice and the proliferative response was measured by [^{3}H]-TdR incorporation on day 4. Titered concentrations of rD-mPGPtide (50 to 200 μmol/L) were added to appropriate wells in order to test the inhibitory properties of the peptide. As shown in Fig 1, the addition of rD-mPGPtide inhibited proliferation in a dose-dependent manner. Nearly 90% inhibition of the proliferative response was observed with the addition of 200 μmol/L of rD-mPGPtide and the 50% inhibitory concentration (IC_{50}) was 50 to 100 μmol/L. Furthermore, the presence of 100 U/mL of recombinant human IL-2 in the cultures failed to re-establish the proliferative response. It appeared that rD-mPGPtide could inhibit alloreactivity generated by this MHC-haploidentical strain combination in vitro, and thus had the potential to inhibit such immune responses in vivo, as well.

rD-mPGPtide inhibits acute GVHD directed across a MHC barrier. An acute form of GVHD was induced in lethally irradiated (950 cGy) (B6CB)F_1 mice by IV administration of 5×10^6 (B6D2)F_1 T cells along with 2×10^6 (B6D2)F_1 ATBM. In this GVHD model, the untreated mice exhibited 80% fatality by day 20 posttransplant with a MST of 13 days (Fig 2). Transplanted mice were also administered three different regimens of rD-mPGPtide treatment, including: (1) daily injections (0.5 mg/injection) from days 0 to 6 post-BMT; (2) alternate days during this same time period (days 0, 2, 4, 6); and (3) every third day (days 0, 3, 6). Treatment with these regimens of rD-mPGPtide increased the MST of these mice to 32, 30, and 28 days, respectively. The observed increases were statistically significant as compared with the untreated group ($P < .03$ for all of the rD-mPGPtide–treated groups), although there was little difference between the three different regimens among themselves ($P > .05$). With time, all of the peptide-treated mice that received donor T cells eventually succumbed to GVHD. Both $CD8^+$ and $CD4^+$ T cells are likely to play a role in the development of GVHD in this strain combination due to the fact that the donor and recipient mice differ at both class I and class II MHC loci. Since the donor T cell subsets were unseparated, there was a potential development of a $CD8^+$ T-cell–mediated GVHD, against which the rD-mPGPtide would be expected to have little effect.

rD-mPGPtide inhibits acute GVHD mediated by MHC allogeneic $CD4^+$ T cells. To specifically evaluate the effect of rD-mPGPtide on $CD4^+$ T cells during the GVHD response, an acute form of GVHD was again induced in irradiated (950 cGy) (B6CB)F_1 mice by administration of 1×10^6 (B6D2)F_1 $CD4^+$ T cells along with 2×10^6 (B6D2)F_1 ATBM. As shown in Fig 3, the MST for those mice left untreated was 25 days. Mice treated with rD-mPGPtide on days 0, 3, and 6 exhibited a significant increase in the MST to >60 days post-BMT ($P < .02$). In this case, 82% of the mice treated with rD-mPGPtide survived for the duration of the experiment, as compared with 27% of the untreated GVHD mice and 91% of the control mice transplanted with only ATBM ($P < .02$). A single injection of rD-mPGPtide also resulted in a significant increase in the MST ($P < .05$) with 80% of the mice surviving past 60 days. A cyclized control scrambled peptide (Scr-PGPtide) was also tested (0.5 mg administered on days 0, 3, and 6) to ensure specificity of the rD-mPGPtide and did not significantly affect survival as compared with the untreated mice ($P > .99$), with only 33% surviving past 60 days (Fig 3). The body weights of surviving animals at the conclusion of the experiment (day 60) exhibited little differences between groups: 30.0 ± 1.0 g for the ATBM group, 27.9 ± 2.4 g for the GVHD group, 26.6 ± 5 g for the rD-mPGPtide day 0,3,6-treated group, 28.1 ± 1.3 g for the rD-mPGPtide day 0-treated group, and 26.6 ± 3.3 g for the Scr-PGPtide–treated group. These data suggested that a chronic form of GVHD was absent in these surviving mice. To examine the possibility that regulatory cells were being generated during peptide treatment, 1.25×10^7 spleen and LN cells from rD-mPGPtide–treated (B6CB)F_1, mice more than 80 days posttransplantation of 10^6 (B6D2)F_1 $CD4^+$ T cells were transferred to de novo-irradiated (B6CB)F_1 mice undergoing GVHD by the same conditions. No enhancement of survival of the mice receiving adoptively transferred lymphoid cells (MST = 16 days; percent survival = 20%) was observed, as compared with the GVHD mice receiving just donor $CD4^+$ T cells (MST = 13 days; percent survival = 0%).

Histopathological analysis. To evaluate the peptide's effect on the clinical manifestation of GVHD in the target tissues, histology sections were prepared 27 days post-BMT from rD-mPGPtide–treated or untreated mice undergoing GVHD. Tissue samples were taken from ear skin and the gastrointestinal tract and examined for morphological changes such as swelling, cellular damage, and the presence of inflammatory infiltrates. In the ear skin and small intestinal tissue of the untreated GVHD mice (Fig 4B and E) there was significant inflammatory infiltration in comparison with the ATBM controls (Fig 4A and D). Numerous dyskeratotic and necrotic cells were also observed in the epidermal layers

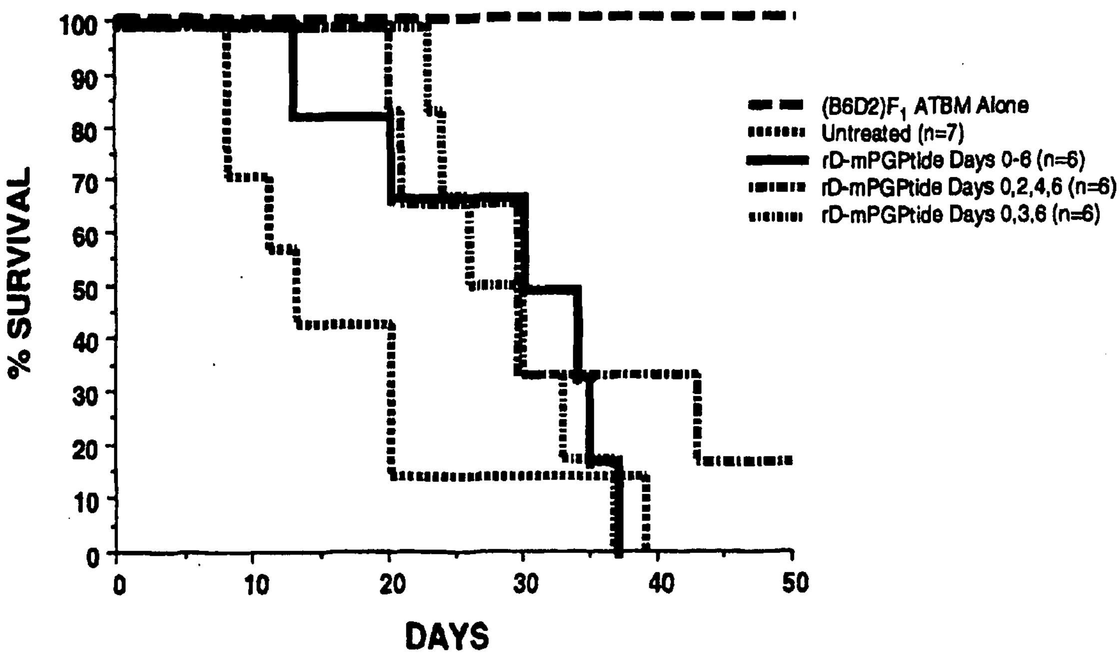

Fig 2. Survival of mice undergoing GVHD across a MHC-haploidentical barrier is enhanced by varying treatments with rD-mPGPtide. (B6CB)F_1 mice were lethally irradiated (950 cGy) and transplanted with allogenelc (B6D2)F_1 ATBM (2×10^6) cells alone, or with unseparated donor T cells (5×10^6). Recipients were either left untreated (positive GVHD control), injected IV with rD-mPGPtide (0.5 mg) daily, every other day, or every third day between days 0 and 6. The data are representative of three separate experiments and the numbers of mice used per group are indicated in the figure.

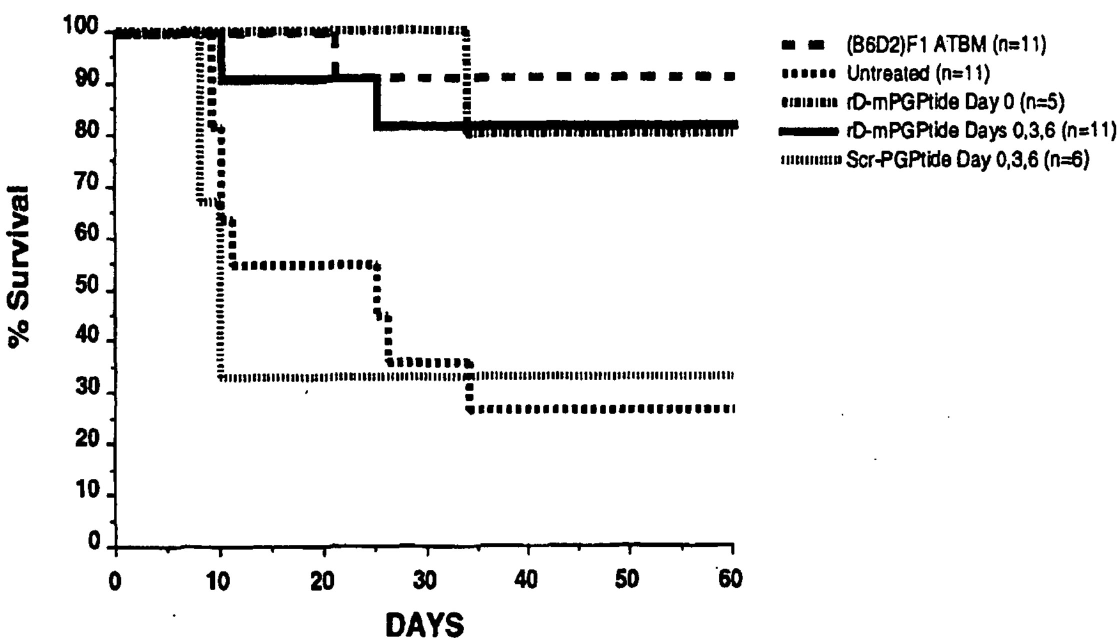

Fig 3. Survival of mice undergoing CD4$^+$ T-cell–mediated GVHD across a MHC-haploidentical barrier is enhanced by treatments with rD-mPGPtide. (B6CB)F_1 mice were lethally irradiated (950 cGy) and transplanted with allogeneic (B6D2)F_1 ATBM (2×10^6) cells alone, or with CD4$^+$ T cells (1×10^6). The rD-mPGPtide (0.5 mg) was administered IV on either day 0 alone or on days 0, 3, and 6. Scr-PGPtide (0.5 mg) was injected on days 0, 3, and 6 and had no significant effect on survival. Data are pooled from two separate experiments and the total numbers of mice used per group are indicated in the figure.

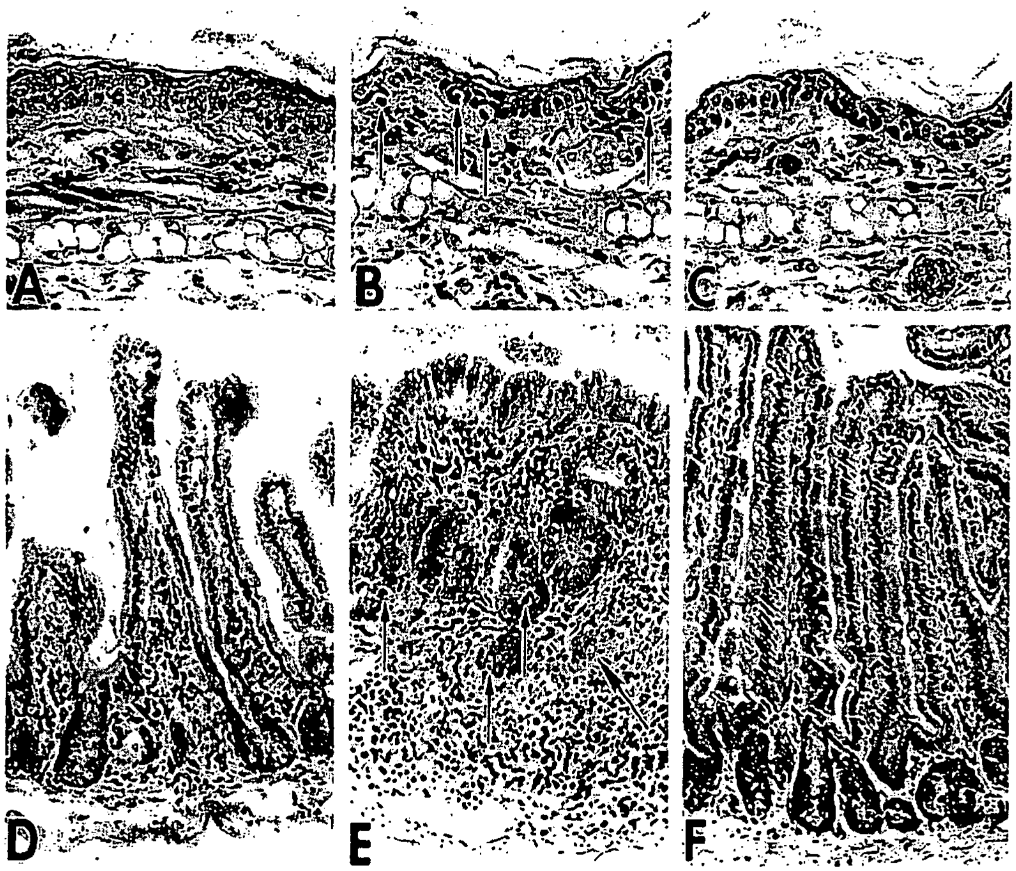

Fig 4. Ear skin (A through C) and small intestine (D and E) of irradiated (950 cGy) (B6CB)F$_1$ mice 27 days after the transplantation of either (B6D2)F$_1$ ATBM (2×10^6) alone (A and D) or with 1×10^6 (B6D2)F$_1$ CD4$^+$ T cells, and left untreated (B and E) or rD-mPGPtide-treated (0.5 mg) on days 0, 3, and 6 (C and F). Two mice per group were examined. The epidermal layer and intestinal epithelium of mice receiving only ATBM (A and D) were devoid of cellular injury, whereas numerous dyskeratotic and necrotic cells (arrows) were observed in the untreated positive GVHD controls (B and E). Also note the cellular infiltrate in lamina propria of positive control intestine (E). Peptide-treated animals failed to exhibit significant skin (C) and gut (F) pathology, and resembled ATBM controls. (Final magnification: A-C, ×800; D-F, ×500).

of both the skin and gut, suggesting GVHD-related cell death. In contrast, the tissue samples from the rD-mPGPtide-treated mice (Fig 4C and F) exhibited limited inflammatory infiltrates and significantly fewer dyskeratotic cells. The differences between the groups were quantitated by counting the number of dyskeratotic cells per linear millimeter of epidermis (Fig 5). The samples from peptide-treated mice displayed a two- to three-fold decrease in the frequency of dyskeratotic cells as compared with those from the GVHD control mice. These combined data suggested that prophylactic treatment of transplanted mice with rD-mPGPtide significantly reduced the clinical manifestations of GVHD, as evidenced by histological sampling.

Effect of rD-mPGPtide on activation antigen expression in vivo—day 4 TDL. To begin investigating the mechanism by which rD-mPGPtide prevents the onset of GVHD, we examined the cell surface of CD4$^+$ T cells from transplanted mice for the expression of activation antigens including IL-2-receptor (IL-2R; CD25), Fas (CD95), and Transferrin-receptor (Tr-R; CD71). Irradiated (950 cGy) (B6CB)F$_1$ mice were transplanted with 10^7 (B6D2)F$_1$ CD4$^+$ T cells, and on day 4 posttransplant their thoracic ducts were cannulated,

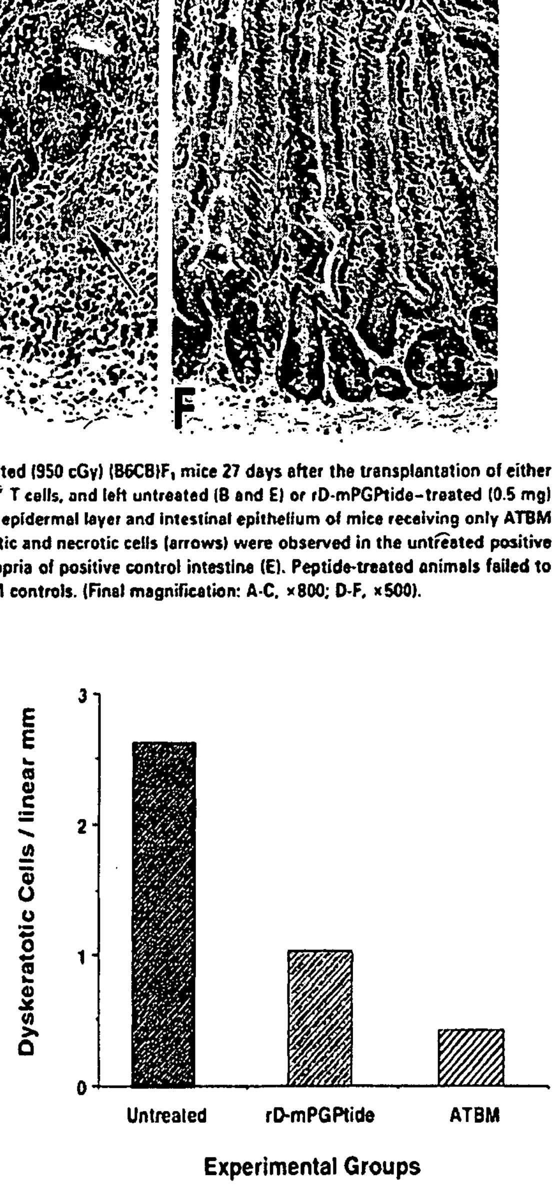

Fig 5. Dyskeratotic cells in the epidermal layer of ear skin were quantitated (#/linear mmol/L) for the same tissue samples as described in Fig 4, A-C. Approximately 10 linear mmol/L were counted in each ear specimen (two mice per group).

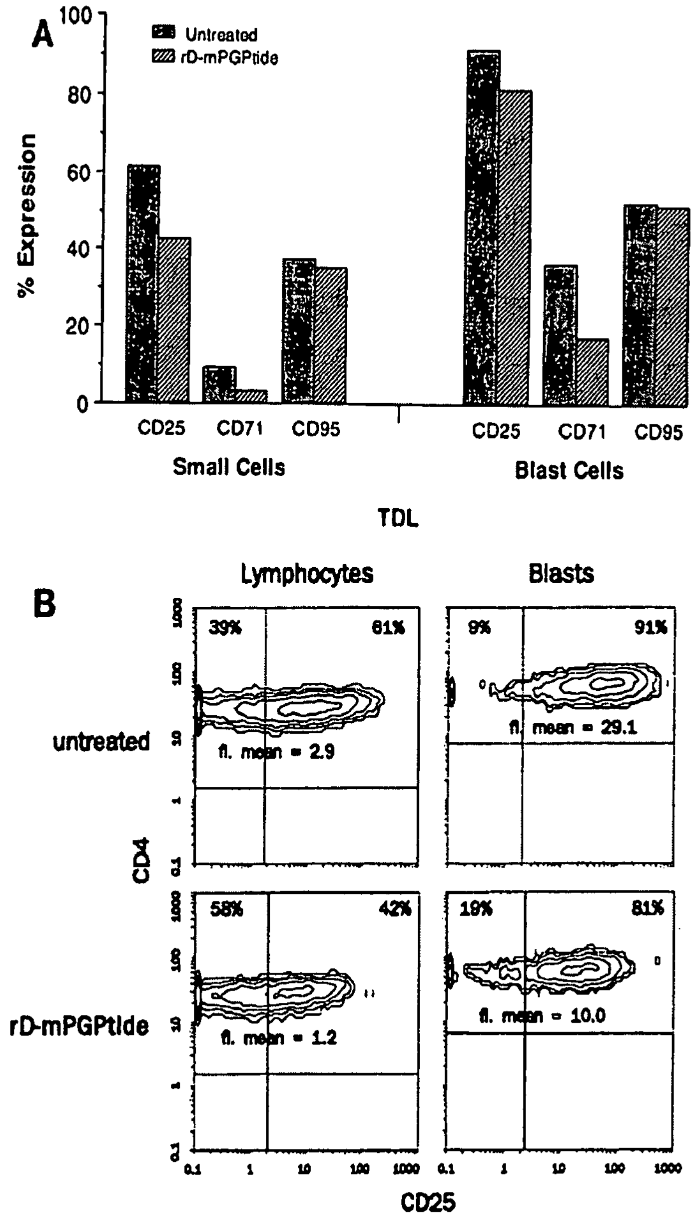

Fig 6. Activation phenotype of TDL cells collected from mice undergoing GVHD is altered by treatment with rD-mPGPtide. (B6CB)F₁ mice (five mice per group) were lethally irradiated (950 cGy) and transplanted with allogeneic (B6D2)F₁ CD4⁺ T cells (1 × 10⁷). Recipients were either left untreated or rD-mPGPtide (0.5 mg) was administered IV on days 0 and 3. Mice were cannulated on day 4 and TDL cells collected over an 8- to 10-hour period. (A) Expression of CD25, CD71, and CD95 as a percentage of the small lymphocytes and the blasting lymphocytes. (B) Flow cytometric profile of the CD4⁺ TDL cells for expression of IL-2 receptor (CD25). The negative control samples for the control and peptide-treated lymphocytes were 0% positive for CD25 with a mean fluorescence of 0.153 and 0.158, respectively. The negative control samples for the control and peptide-treated blast cells were 9% and 7% positive for CD25, respectively, with a mean fluorescence of 0.226 and 0.232, respectively.

TDL were collected over an 8 to 10 hour period, and flow cytometric analysis was performed on the retrieved cells. Transplanted mice (3 per group) were either left untreated or were injected IV with 0.5 mg rD-mPGPtide on days 0 and 3. The TDL collected from each group were exclusively donor-type $CD4^+$ T cells; however, the flow yield of TDL collected from peptide-treated mice was significantly less than those of the untreated mice, 4×10^5 cells/mL versus 7×10^5 cells/mL, respectively. Both experimental groups of TDL contained a high percentage of blast-like cells (26% to 28%), and were analyzed as a separate population from the remaining small lymphocytes. As shown in Fig 6A, the percentage of TDL cells expressing the activation antigen CD25 was very high (90% for blast cells and 60% for non-blast cells) in the mice undergoing GVHD, suggesting that the allogeneic donor $CD4^+$ T cells were reacting to the host alloantigens. Treatment of the mice with rD-mPGPtide resulted in a 30% reduction in the percentage of non-blast cells expressing IL-2R and a 10% reduction in the percentage of blast cells expressing IL-2R. More notable, were the changes in the mean antigen expression of IL-2R on both the blast cells and the non-blast cells. Nearly a three-fold reduction was seen for both populations (Fig 6B). Tr-R expression was also affected in a similar manner; however, Fas appeared to be expressed at a level of 50% in both cell populations and remains unchanged in the peptide-treated group (Fig 6A).

Alloreactivity of GVHD TDL cells. As a measure of alloreactivity, MLR cultures were established using the TDL collected above as responder cells. These cultures were stim-

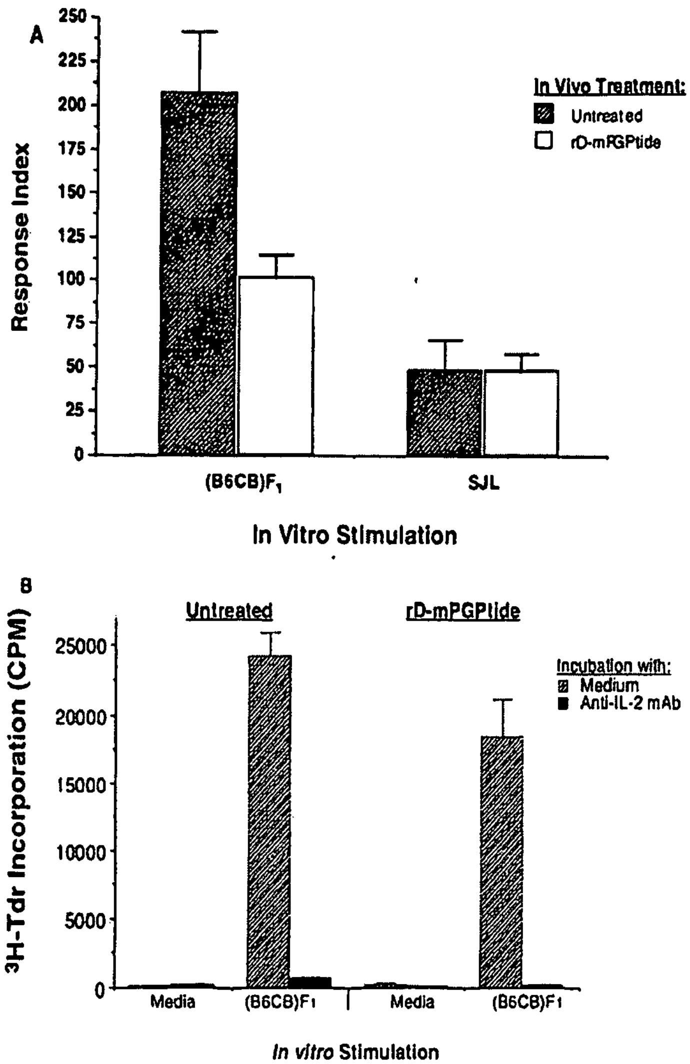

Fig 7. Alloreactivity of TDL cells collected from mice undergoing GVHD is reduced by treatment with rD-mPGPtide. The TDL cells were collected in the same manner as described in Fig 6 and the data are representative of three similar experiments. (A) MLR proliferation responses to irradiated host $(B6CB)F_1$ splenocytes were performed as described in the Materials and Methods section. Data is expressed as the response index of proliferation ± SEM. (B) CTLL proliferation supported by culture supernatants from TDL alloresponses. Data is expressed as CPM [^{3}H]TdR incorporation ± SEM.

ulated with either irradiated (15 Gy) $(B6CB)F_1$ splenocytes or media alone for 48 hours and pulsed with [^{3}H]TdR as a measure of proliferation for the final 24 hours. As shown in Fig 7A, [^{3}H]TdR incorporation by TDL from untreated control mice was enhanced in response to $(B6CB)F_1$ stimulator cells, as compared with the syngeneic $(B6D2)F_1$ stimulator cells. TDL collected from the rD-mPGPtide-treated mice exhibited a 50% reduction in proliferation when incubated with the allostimulator cells as compared with the untreated TDL proliferation ($P < .01$). Furthermore, proliferation responses to third party alloantigens (SJL spleen cells) remains intact following peptide treatment ($P > .05$). These results suggested that proliferative responses to alloantigen are impaired in the peptide-treated mice. To further analyze this proliferative response, culture supernatants from another representative experiment were removed after 24 hours and analyzed for cytokine content by a CTLL bioassay. As shown in Fig 7B, supernatants from the untreated control TDL cultured with media alone were not capable of supporting CTLL proliferation. Incubation of TDL with $(B6CB)F_1$ stimulator cells significantly enhanced the CTLL proliferation of the culture supernatants. This proliferation was neutralized by anti-IL-2 MoAb suggesting little or no IL-4 production by the TDL in response to the alloantigen stimulation. The TDL from the rD-mPGPtide-treated mice demonstrated slightly reduced cytokine production ($P > .01$), which was neutralized by anti-IL-2 MoAb as well, as compared with the untreated TDL. These results suggested that the proliferation defect of the TDL from peptide-treated mice may not be entirely accounted for by a reduced capacity to produce IL-2.

DISCUSSION

In this report, we have described a novel therapeutic approach for the treatment of murine GVHD across a MHC barrier using a peptide analog of the CDR3 region of the murine CD4 molecule, rD-mPGPtide. Experiments performed both in vitro and in vivo have demonstrated a potential for rD-mPGPtide to affect immune responses to alloantigens. The inhibition of the alloreactive immune response was indicated in vivo primarily by the prolongation of survival of mice undergoing GVHD and reduced tissue destruction in

these mice. This enhanced survival was evident for GVHD induced by both unseparated T cells and purified $CD4^+$ T cells (Figs 2 and 3). Even a single injection of rD-mPGPtide at the time of transplant was effective in delaying the onset of disease mediated by $CD4^+$ T cells. The protective response of the rD-mPGPtide appears to be specific as the control scrambled peptide failed to exhibit significant enhancement of survival as compared with untreated mice in this model (Fig 3).

Upon comparing the two experimental conditions tested, unseparated T cells and purified $CD4^+$ T cells, it is apparent that the rD-mPGPtide was more effective in preventing the GVHD induced by the purified $CD4^+$ T cells. We hypothesize that in the case of the unseparated T cells, the rD-mPGPtide has limited effect on the $CD8^+$ T-cell–mediated component of GVHD directed to MHC class I antigens, a large portion of which may actually be CD4-independent.[13] Yet, even under these arduous conditions, the CD4-CDR3 peptide could prolong the survival time of recipient mice by at least 2 weeks.

Based on the animal survival data, it is apparent that the alloreactive $(B6D2)F_1$ $CD4^+$ T cells from the donor are less potent at mediating GVHD in the rD-mPGPtide–treated recipient. The anti-host specific donor T cells, themselves, are most likely rendered dysfunctional in their ability to respond to alloantigen. This is supported by several observations, including the reduction of in vitro alloreactivity and activation antigen expression by the positively selected TDL collected from mice treated with rD-mPGPtide. In vitro alloresponses and cytokine production were evident in the TDL of the mice undergoing GVHD (Fig 7, A and B); however, the TDL from the peptide-treated mice exhibited a 50% reduction in the proliferative response to alloantigen, but only a 15% reduction in IL-2 production. This reduced proliferative capacity could be due to either an inability of the T cells to recognize alloantigen or the presence of fewer alloreactive T cells due to deletion and/or inhibition of expansion. However, these results suggest that the observed inhibition of proliferation is relatively independent of IL-2 production. Furthermore, expression of all three activation antigens tested (IL-2R, Tr-R, and Fas) were increased on the TDL of GVHD mice (Fig 6A), whereas the TDL from the rD-mPGPtide–treated mice exhibited a marked reduction in the expression of IL-2R and Tr-R, yet no reduction in the expression of Fas. These findings may hold a clue to the mechanism by which this CD4-CDR3 peptide analog mediates inhibition of GVHD. It has been shown previously that the expression of Fas is required for the induction of T-cell apoptosis on incomplete activation, while the lack of Fas expression leads to T-cell anergy.[29] The continued high expression of Fas on the TDL from the peptide-treated mice may allow for the induction of apoptosis of the allo-specific T cells on incomplete activation in the presence of peptide. Further investigation into the peptide mechanism is necessary to clarify this issue.

Several recent reports propose that alterations in the immune response can be induced which generate a protective effect against various immunologically based disorders. These approaches include the polarization in cytokine production from Th1-like to Th2-like phenotypes,[30,31] potentiation of other specific cytokines (eg, TGFβ[32,33]), inhibition of T-cell trafficking to target organs,[34-38] or the generation of non-lethal regulatory cells in vivo.[39,40] The switching of cytokine phenotype from Th1 to Th2 has been reported to be induced by several agents including cytokines[41] and anti-CD4 MoAb.[42,43] The induction of a Th1 immune response in GVHD has been correlated with lethality and a switch in the response to a Th2 phenotype has been shown to significantly prolong survival.[30,31] In this regard, we investigated whether or not rD-mPGPtide was inducing such a cytokine switch leading to protection of the transplanted mice. TDL taken from these peptide-treated mice demonstrated no appreciable difference in the IL-2 and IL-4 production profiles when stimulated ex vivo by alloantigen (Fig 7B). Therefore, our data do not support a rD-mPGPtide–induced cytokine switch phenomenon in alloreactive T cells during the development of GVHD to account for the inhibitory effects of the peptide.

In order for the T cells to cause GVHD, they must be able to traffic to the appropriate target organs including the skin, gut, and liver. Inhibiting the trafficking of these alloreactive T cells could also lead to a reduction in the symptoms of GVHD. The expression of adhesion molecules such as CD44, ICAM-1, VCAM-1, and LFA-1 on the surface of T cells play an important role in controlling the trafficking of these cells to their target tissues.[35,44,45] The histopathological analysis of mice undergoing GVHD suggested that rD-mPGPtide was inhibiting the infiltration and subsequent destruction of epithelial tissue in the skin and gastrointestinal tract (Fig 4). In addition to the possible inhibition of proliferation and expansion of alloreactive T cells, this apparent inhibition of trafficking could also be a result of decreased expression of adhesion molecules on the surface of the activated T cells or insufficient upregulation of the ligand molecules on vascular tissues. These possibilities are currently under investigation.

The generation of regulatory cells controlling immune responses in various mouse models of autoimmunity has been previously reported.[39,40,46] Adoptive transfer of these cells from protected animals to unprotected autoimmune-prone animals can lead to protection from disease and is one way of demonstrating the existence of these regulatory populations of cells. We examined this possibility by transferring spleen and lymph node cells from rD-mPGPtide–treated $(B6CB)F_1$ mice more than 80 days posttransplantation of 10^6 $(B6D2)F_1$ $CD4^+$ T cells to de novo-irradiated $(B6CB)F_1$ mice undergoing GVHD by the same conditions. We observed no enhancement of survival of the mice receiving adoptively transferred lymphoid cells as compared with the GVHD mice receiving just donor $CD4^+$ T cells. Thus, it appears that the generation of a regulatory population of cells is not responsible for the observed protective effect of rD-mPGPtide in these mice undergoing GVHD.

As a therapeutic modality, the rD-mPGPtide peptide mimic of the CD4-CDR3 molecular site appears to be an effective agent for the prevention of GVHD. We believe that the peptide primarily affects the alloreactive T cells that are being activated early after transplantation, rendering them

incapable of inducing GVHD. Results from recent EAE studies[47] with the peptide provide strong evidence that inhibition is highly specific for CD4$^+$ T-cell responses to antigens present at the time of exposure to the rD-mPGPtide. One, 2, or 14 days after treatment, there is no diminution in any of the lymphoid cellular compartments, including the CD4$^+$ T-cell subset. In addition, lymph node T cells are fully functional in their capacity to respond to both recall antigens and to third-party alloantigens. The half-life retention of peptide in serum in mice is approximately 25 minutes and responsiveness to any type of antigen stimulation is significantly inhibited for up to 6 hours after administration, but has virtually no effect by 12 hours. Noting this short window of effect of the CD4-CDR3 peptide, it is a most intriguing possibility that if administered only within the first week of transplant, the peptide could leave the remaining non-alloreactive CD4$^+$ T-cell population intact for subsequent development of responses to opportunistic infections or potential leukemic relapse. Further studies are planned to clarify these issues.

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